CHARACTERIZATION OF THE CHEMOTAXIS SYSTEM OF THE ENDOSYMBIOTIC BACTERIUM R*HIZOBIUM LEGUMINOSARUM* BV. *VICIAE* STRAIN 3841

A Dissertation
Presented to
The Academic Faculty

by

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In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in the
School of Biology

Georgia Institute of Technology
December 2007

CHARACTERIZATION OF THE CHEMOTAXIS SYSTEM OF *RHIZOBIUM LEGUMINOSARUM* BV. *VICIAE* STRAIN 3841

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ACKNOWLEDGEMENTS

I would like to thank everyone who contributed to this work and supported me throughout my graduate career. This journey has taken me to the bottom of the ocean in the Gulf of Mexico and to mountain tops in Canada. In pursuit of this degree, I have had the fortune of traveling to other countries and have made friends with people from around the world. It has been a privilege to participate in and contribute to science, that most democratic of all human endeavors, and it would not have been possible without the help, love, and support of my family, friends, collaborators, and colleagues.

I wish to thank members of my committee past and present. I would especially like to thank my co-advisors Gladys Alexandre and Igor Zhulin. Igor has been a true mentor who has taught by example, and Gladys has an infectious enthusiasm and passion for experimental biology that has been an inspiration for me.

I would most like to thank my partner in life and fellow biologist, Star, who has made enormous sacrifices to see this goal through. Her constant support and encouragement have made accomplishing this goal not only possible but worthwhile.

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LIST OF SYMBOLS OR ABBREVIATIONS

bp	base pairs
°C	degrees Celsius
Ca^{2+}	calcium
CCW	counter clockwise
CW	clockwise
DNA	deoxyribonucleic acid
Gm	gentamicin
G+C	guanidine plus cytosine content
kb	kilobases
Km	kanamycin
kV	kilovolts
LB	Luria-Bertani media
µm	micrometers
TCS	two-component system
µl	microliter
MCPs	methyl-accepting chemotaxis protein
min	minute
MiST	Microbial Signal Transduction database
ml	milliliter
mM	millimolar

MW	molecular weight
NCBI	National Center for Biotechnology Information
nmol	nanomolar
OD_{600}	optical density, 600 nm wavelength
PCR	Polymerase Chain Reaction
rpm	revolutions per minute
sec	seconds
SMART	Simple Modular Architecture Research Tool
Sm	streptomycin
Sp	spectinomycin
TEM	transmission electron microscopy
Tc	tetracycline
TY	tryptone yeast media
VMM	Vincent's Minimal Media
wt/v	weight per volume

SUMMARY

Chemotaxis is the process by which motile bacteria navigate chemical gradients in order to position themselves in optimum environments for growth and metabolism. Sensory input from both the external environment and the internal cellular environment are sensed by chemotaxis transducers and transduced to a two-component system whose output interacts with the flagellum thereby regulating motility.

Chemotaxis has been implicated in establishing the endosymbiotic relationship between the motile alpha-proteobacterium *Rhizobium leguminosarum* biovar *viciae* and its host *Pisum sativa*, the pea plant. An approach combing bioinformatical sequence analysis, molecular genetics, and behavioral analysis was used to characterize the chemotaxis system of *R. leguminosarum* and determine its contribution to this bacterium's lifestyle.

A genome search revealed the presence of two chemotaxis gene clusters, *che1* and *che2*. Homologs of each che cluster are major chemotaxis operons controlling flagellar motility in other bacterial species. For this reason we sought to determine the contribution of each che cluster to chemotaxis in *R. leguminosarum*. We found that while both *che* clusters contribute to the regulation of motility, *che1* is the major *che* cluster controlling chemotaxis. Using competitive nodulation assays we determined that *che1*, but not *che2*, is essential for competitive nodulation.

The major che cluster, *che1*, encodes a chemotaxis transducer, IcpA-*Rl*, with a globin coupled sensor domain. Chemotaxis transducers with a globin coupled sensor domain comprise a large class of proteins found in bacteria and archaea. These proteins

have been shown to bind heme and sense oxygen and are therefore termed HemATs for heme-binding aerotaxis transducers. However, sequence analysis of IcpA-*Rl* reveals that it lacks the requisite amino acid residues for heme-binding and is therefore unlikely to sense oxygen. We present evidence that IcpA-*Rl* is likely an energy transducer and represents a novel function of the globin coupled sensor domain in sensing energy related parameters.

CHAPTER 1

INTRODUCTION

In order to optimize physiology in constantly changing environmental conditions, every living organism must continuously sense and respond to its environment. Despite their small size, single cell bacteria can sense and respond to a wide range of environmental stimuli including osmolarity, pH, temperature, light, and numerous chemical ligands (Armitage, 1999; Wadhams and Armitage, 2004). Chemotaxis is one way motile bacteria respond to their environment. Bacteria use chemotaxis to direct their motility in chemical gradients towards increasing concentrations of attractants or away from increasing concentrations of repellents. This allows cells to position themselves in environmental niches best suited for their growth and metabolism.

A Two-component System Controls Chemotaxis

Motile bacteria achieve chemotaxis by executing a biased random walk. Using flagellar-based motility bacteria can only either swim smoothly in a single direction or execute a "tumble" which reorients the cell allowing it to resume swimming in a different random direction. Bacteria are able to navigate chemical gradients by biasing the rate at which tumbling occurs. Movement in the direction of an increasing concentration gradient of an attractant results in suppression of tumbling or smooth swimming. Movement away from an attractant results in an increase in the frequency of tumbling. The converse is true for repellants. This type of sensing is temporal rather than spatial.

The chemical concentration at present is compared to that of a few seconds prior and a decision is made about the difference (Blair, 1995; Falke *et al*., 1997).

At the center of the protein regulatory network that controls chemotaxis lays a two-component system (Falke *et al*., 1997). Two-component systems, TCS, are typically comprised of a membrane bound sensor histidine kinase coupled with a cytoplasmic cognate response regulator (Stock *et al.*, 2000; Hoch, 2000). The histidine kinase possesses an input domain that senses an environmental signal. This causes autophosphorylation at a conserved histidine residue. The phosphorylated histidine kinase can then donate its phosphoryl group to an aspartate residue in the receiver domain of its cognate response regulator. Once phosphorylated the cognate response regulator becomes activated and can then carry out its cellular function via its output domain. TCS can sense a wide range of environmental stimuli and usually result in a change in gene expression although they can regulate other cellular processes (Stock *et al.*, 2000; Hoch, 2000).

Until recently TCS were thought to be the dominant mechanism for prokaryotic signal transduction. However, a recent study analyzing completely sequenced prokaryotic genomes has found that so called one-component systems consisting of a single protein with both input and output domains are both more numerous, ~17,000 versus ~4,000, and more diverse with respect to their input domains than two-component systems (Ulrich *et al.*, 2005). Many one-component systems are thought to sense the internal cellular environment and likely represent the evolutionary precursors of two-component systems (Ulrich *et al.*, 2005).

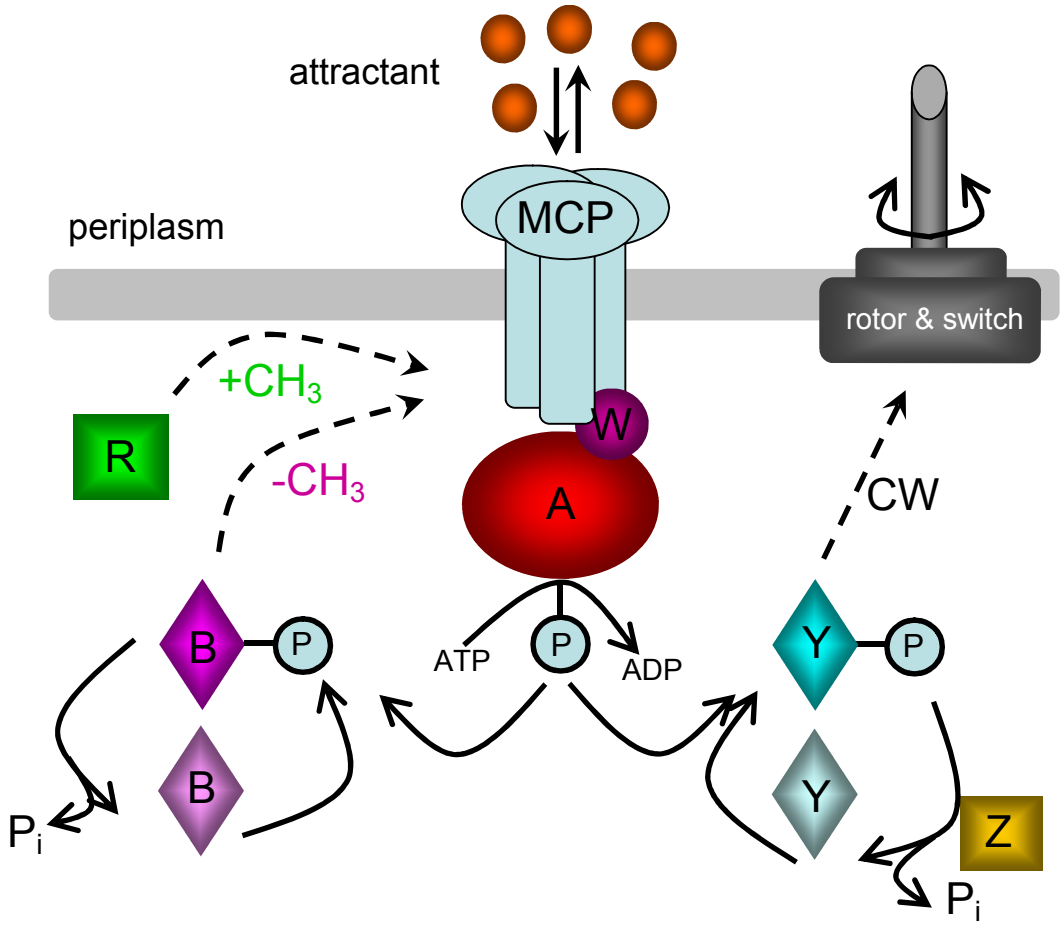

Figure 1.1 Schematic diagram of the *E. coli* chemotactic signal transduction pathway. Adapted from Parkinson (2003). Methyl-accepting chemotaxis proteins, MCPs, transduce signals from chemoeffectors in the periplasmic space thereby modulating the activity of the histidine kinase, CheA, which is linked to the MCPs by CheW. CheA phosphorylates the response regulators CheB and CheY whose phosphorylated forms interact with the MCPs and the flagellar motor respectively. CheR also interacts with the MCPs whereas the dephosphatase CheZ dephosphorylates CheY. See text for a complete discussion.

A schematic diagram of the *E. coli* chemotactic signal transduction pathway adapted from Parkinson (2003) is provided in Figure 1.1. The two-component system at the core of the chemotactic system consists of CheA, the sensor histidine kinase, and CheY its cognate response regulator (Bren and Eisenbach, 2000). This two component system processes signals from so called methyl-accepting chemotaxis proteins or MCPs that are embedded in the cytoplasmic membrane and sense environmental signals. An N-terminal sensory domain protrudes into the periplasm and receives signals that are then transmitted across the cytoplasmic membrane to the C-terminal signaling domain (Falke and Hazelbauer, 2001). MCPs are arranged into dimers that are clustered into larger arrays of trimers of dimers or so called "forests of dimers" that are localized to the cell poles (Maddock and Shapiro, 1993; Bray *et al.*, 1997; Alexander and Zhulin, 2007).

MCPs are connected to the histidine kinase CheA via the linker protein CheW. When CheA is activated it autophosphorylates at its conserved histidine residue and in turn phosphorylates its cognate response regulator CheY. Phosphorylated CheY, CheY-P, then freely diffuses through the cytoplasm and binds FliM, the switch protein of the flagellar motor thus effecting a change in the direction of rotation. This change in the rotational direction of the flagellum alters its morphology causing the bundle of flagella that propels the cell forward to dissociate causing a tumbling event. The signal is ultimately terminated by dephosphorylation of CheY-P by the phosphatase CheZ.

CheB and CheR are required for sensory adaptation

Bacteria are able to sense and navigate chemical concentration gradients over a wide range of concentrations from nanomolar to millimolar. Since bacteria utilize a

temporal sensing mechanism instead of sensing the absolute concentration, they must be able to adapt to the current level of a stimulus. This enables them to respond to future changes in a stimulus concentration within a spatial gradient. CheB and CheR comprise the system allowing for adaptation to attractant or repellent concentrations (Stock and Surette, 1996).

CheB is a methylesterase, and CheR is a methyltransferase. CheR is constitutively active, constantly adding methyl groups from S-adenosylmethionine to the conserved glutamate residues at methylation sites on the chemoreceptors. An increase in an MCPs level of methylation results in a suppression of its signaling. CheB counteracts this effect. CheB becomes active after it is phosphorylated by CheA. Once activated, CheB removes methyl groups from MCPs thus restoring their ability to signal.

Attractant binding shifts the receptor towards methylation since attractant binding suppresses phosphorylation of CheA and thus CheB. A decrease in attractant binding results in increased levels of CheA-P thus increasing levels of CheB-P and results in demethylating the receptor. CheB and CheR act in concert allowing cells to adapt to stimuli thereby resetting receptor sensitivity and enabling future sensing of changes in concentration.

Implications for Temporal Sensing of Chemical Gradients

It is often reported that bacteria are too small to sense chemical gradients spatially. While there are some reports of bacteria employing spatial sensing for chemotaxis (Hader, 1987; Thar and Kuhl, 2003), most bacteria employ a temporal sensing mechanism as described above. This could be because temporal sensing is more

efficient for bacteria, despite an estimated effective velocity that is roughly 10% that of the maximum swimming velocity due to the biased random swimming path necessary for temporal sensing (Berg, 1993).

Bacteria tend to inhabit chemically heterogeneous environments. It may not always be advantageous for a bacterium to simply swim up the gradient of the strongest chemoattractant. For instance, two attractant gradients could be in opposite directions making a position in between optimum (Alexandre *et al*., 2004). Marine sediments with opposite gradients of oxygen and sulfide represent an example of this scenario (Jorgensen, 1982). In this situation it would be disadvantageous for a sulfide oxidizing bacterium to merely follow a single attractant gradient: either oxygen or sulfide. Instead, it is more advantageous for this bacterium to seek a niche with a balance of oxygen, sulfide, and other chemoattractants such as carbon sources optimized for its individual physiology. This is particularly true provided that energy taxis is the dominant behavior of some organisms (Alexandre *et al.*, 2000; Alexandre and Zhulin, 2001; Alexandre *et al.*, 2004).

Bacteria Also Respond to Their Environment Via Energy Taxis

In addition to the classical ligand binding chemotaxis described above, many bacteria are able to perform energy taxis. In energy taxis cells monitor their internal energy status and bias their motility accordingly. Metabolism is required for energy taxis, therefore the signal for energy taxis is not a chemical ligand per se, but it derives from the electron transport system or proton motive force. Because of this any chemical that has an affect on metabolism will elicit an energy taxis response. For example,

carbon sources that are electron donors and electron acceptors such as oxygen, nitrate, or nitrite will elicit energy taxis responses (Taylor *et al*., 1999; Alexandre and Zhulin, 2001).

Energy taxis has been studied extensively in *E. coli* (Taylor *et al*., 1999). The MCPs Aer and Tsr independently transduce signals for oxygen, redox, and energy signals (Rebbapragada *et al*., 1997; Greer-Philips *et al*., 2003). Aer bears an FAD binding PAS domain that monitors changes in the electron transport system whereas Tsr is a classical ligand binding chemoreceptor that senses serine, but also has the ability to sense intracellular energy levels probably by monitoring the proton motive force (Rebbapragada *et al*., 1997; Greer-Philips *et al*., 2003).

In some species, such as *Azospirillum brasilense*, energy taxis is the dominant behavior (Alexandre *et al*., 2000), and energy taxis is thought to be widespread among bacteria and might play an important role in structuring microbial communities (Alexandre *et al*., 2004).

Many Chemotaxis Systems Have Additional Components

Analysis of chemotaxis systems from completely sequenced bacterial genomes suggests that gene loss is a driving force in the evolution of the chemotaxis system (Wuichet and Zhulin, unpublished). The conserved set of CheAWYBRZ proteins and five MCPs comprising the chemotaxis protein network of *E. coli* is a streamlined version resulting from the evolutionary loss of chemotaxis proteins still present in the chemotaxis systems of other organisms.

There are several additional proteins known to function in chemotaxis. *Bacillus subtilis* is another model organism for chemotaxis because it possesses a copy of most (but not all) chemotaxis proteins that have been found (Szurmant and Ordal 2004). Similar to *E. coli,* it possesses a single chemotaxis operon, but it encodes the additional chemotaxis accessory proteins CheC, CheD and CheV. CheC functions in adaptation and signal termination. CheD is a deamidase that functions in receptor maturation by deamidating specific glutamine residues of MCPs. This function is carried out by CheB in *E. coli*. CheV also has a role in adaptation and in coupling CheA to the chemotaxis transducers (Szurmant and Ordal, 2004).

Many Bacteria Have Multiple Che Operons and Some che Operons Regulate Other Cellular Functions

In addition to accessory proteins many bacteria also possess multiple chemotaxis operons encoding homologues of the *E. coli* and *B. subtilis* chemotaxis proteins (Szurmant and Ordal, 2004). The chemotaxis operons in these organisms can regulate flagellar and pili-mediated motility as well as perform other cellular functions such as gene regulation. *Pseudomonas aeruginosa* illustrates the complexity of some chemotaxis systems because many different chemotaxis genes from different gene clusters are involved in its chemotaxis system.

The *P. aeruginosa* genome encodes 20 che genes in five separate clusters as well as 27 MCPs. Components from three che clusters, cluster I, cluster II, and cluster IV, contribute to flagellar motility (Kato *et al*., 1999; Masduki *et al*., 1995; Ferrandez *et al*., 2002; Hong *et al.*, 2004b), and one of these may also regulate expression of pathogenicity

genes (Schuster *et al.*, 2004). A fourth che cluster, cluster III, is involved in twitching (pili-mediated motility) (Darzins, 1993; Darzins, 1994), and a fifth, cluster V, regulates cell surface factors involved in autoaggregation (D'Argenio *et al*., 2002).

Rhodobacter sphaeroides encodes three distinct che operons. Chemosensory components from at least two operons are required for chemotaxis (Porter and Armitage, 2002; Porter and Armitage, 2004). The components from these operons localize to different parts of the cell, the pole and the cytoplasm. Interestingly, signals for chemotaxis must be processed through the cytoplasmic cheA demonstrating cross-talk between the different systems.

The δ-proteobacterium *Myxococcus xanthus* displays social behavior, undergoes a complex developmental program, and exhibits two different types of motility, social, S, and adventurous, A. *M. xanthus* has as many as eight chemosensory operons (Wadhams and Armitage, 2004) and requires three chemotaxis operons, dif, frz, and Che4, for its different modes of motility. dif is involved in S motility (Yang *et al*.,2000; Li *et al*., 2003), frz is required for S and A (Ward and Zusman, 1999; Sun *et al*.,2000), and Che4 is involved in S (Vlamakis *et al*., 2004). An additional operon, Che3, is involved in regulating its developmental program (Kirby and Zusman, 2003).

In *Rhodospirillum centenum,* three chemotaxis-like operons have been described, only one of which controls locomotive behavior (Jiang *et al*., 1997). A second chemotaxis operon was found to be involved in cyst formation (Berleman *et al*., 2004; Berleman and Bauer, 2005a), while a third has been implicated in controlling flagellum synthesis (Berleman and Bauer, 2005b).

Azospirillum brasilense possesses four chemotaxis operons. Components from multiple chemotaxis operons are likely involved in controlling chemotaxis in *A. brasilense* (Stephens *et al*., 2006). In addition, one che operon contributes to the regulation of cell size in *A. brasilense* (Stephens, 2006).

The observation that orthologous operons perform different functions in different organisms complicates the prediction of their role in the biology of the organisms based on sequence comparisons alone. For example, *Agrobacterium tumefaciens, Sinorhizobium meliloti,* and *R. sphaeroides* all encode a homologous chemotaxis operon. In the rhizobial species *Agrobacterium tumefaciens* and *Sinorhizobium meliloti* this chemotaxis operon controls chemotaxis and motility, but it does not appear to be a major regulator of chemotaxis under laboratory conditions in *R. sphaeroides* (Armitage and Schmitt, 1997; Wright *et al*., 1998).

Ecological Role and Environmental Regulation of Chemotaxis and Motility

The widespread presence of chemotaxis operons in the complete genomes of bacteria and archaea is evidence of the evolutionary and biological benefits of chemotaxis. Since bacteria inhabit almost every conceivable environmental niche in the biosphere and are capable of undergoing complex cellular differentiation processes in order to live, grow, and divide in these different environments, the ecological role of chemotaxis has long been of interest. Studies are beginning to addressing the question of what role chemotaxis plays in structuring microbial communities and under which environmental conditions chemotaxis and mode of motility provides a competitive advantage (Fenchel, 2002; Alexandre *et al*., 2004).

Some bacteria regulate expression of chemotaxis components to optimize physiology for different growth conditions. For example, the life cycle of *Caulobacter crescentus* alternates between a free-swimming chemotactically competent cell and a nonmotile nonchemotactic stalk cell affixed to a substrate (Alley *et al*., 1993; Ryan and Shapiro, 2003; Skerker and Laub, 2004). *C. crescentus* cells regulate expression of chemotaxis proteins and segregate these proteins during cell division to accomplish this developmental feat. Members of the rhizobiaceae are capable of infecting the roots of various plants and differentiating into nonmotile nitrogen fixing bacteroid cells that have down regulated expression of genes involved in chemotaxis and motility (Becker *et al*., 2004; Yost *et al*., 2004).

Other bacteria regulate their mode of motility to optimize their physiology. Bacteria such as *Vibrio parahaemolyticus* and *Azospirillum brasilense* possess dual flagellar systems, a polar flagellum for swimming and lateral flagella for swarming motility (Merino *et al*., 2006). These bacteria regulate flagellar expression under varying environmental conditions to optimize motility. For example, *A. brasilense* swims via its polar flagellum in soil (Bashan, 1986) but uses its lateral flagella to move along plant roots (Moens *et al*., 1995). *V. parahaemolyticus*, an animal pathogen, uses its polar flagellum to swim, but induces lateral flagella for adherence and colonization of surfaces necessary for infection (Merino *et. al*. 2006).

Moreover, the observation that many bacterial species possess more than 20 chemoreceptor genes (Alexandre *et al*., 2004) has led to speculation that expression of receptors might be fine tuned under different environmental circumstances to optimize chemotaxis (Wadhams and Armitage, 2004). For example, *Pseudomonas aeruginosa*

encodes two different aerotaxis receptor genes, *aer* and *aer-2*. The expression of these two receptors is controlled by two different transcriptional regulators, ANR and RpoS respectively (Hong *et al*., 2004a; Hong *et al*., 2005).

Together these observations demonstrate that chemotaxis and motility mechanisms are regulated to optimize behavioral responses and cellular physiology under varying environmental and growth conditions. However, more work is required to understand how and under what conditions expression of chemotaxis components is regulated to optimize behavior for many organisms.

Chemotaxis and Motility Play a Role in Establishing Host-microbe Relationships

Many bacteria live in close association with a host organism. From the perspective of the host organism these associations range from harmful, pathogenic or parasitic, to commensal in which the host organism is unaffected, to symbiotic or mutually beneficial. Any of these three types of bacterium and host associations could be between a bacterium and a plant, animal, or fungal host. The role of chemotaxis and motility in establishing different types of bacterium/host associations has been studied for several organisms providing interesting and in some cases counterintuitive insights.

In general, chemotaxis plays an advantageous role in establishing bacterium/host relationships allowing bacteria to locate and swim toward hosts. However, since chemotaxis is regulated motility by definition, mutations affecting chemotaxis often alter a cell's motility making it difficult to determine whether effects are due to chemotaxis or motility defects. One example discussed below is *Vibrio cholera* which appears to require motility but not chemotaxis to infect its host.

Despite the common sense notion that chemotaxis would provide an advantage for establishing bacterium/host associations, in many cases non-motile and non-chemotactic mutants are unaffected in establishing infection or symbiosis *in vitro* or i*n vivo* when inoculated alone in the absence of competition. It is therefore necessary to perform competition experiments to observe this advantage experimentally. This has led many researchers to conclude that chemotaxis and motility confer an advantage only under certain environmental conditions.

However, even a minor advantage would be significant in the real world. For example, a typical gram of soil may contain a billion bacterial cells representing 10,000 species or more (Torsvik *et al*., 1990; Curtis and Sloan, 2005; Gans *et al*., 2005). This level of diversity and population density represents enormous competition for limited resources. Therefore, it is likely that even a minor advantage conferred upon a bacterium by chemotaxis and motility will be significant given the extreme diversity and high population density of environments such as the soil. What follows is a brief overview of a few of the best studied bacterium/host associations where the role of chemotaxis has been examined.

Helicobacter pylori

Helicobacter pylori is a human gastric pathogen that infects the stomach and causes gastric ulcers (Marshall *et al.*, 1985). *H. pylori* has a unique chemotaxis system consisting of a CheACheY fusion, CheY, a CheW, and three CheVs. There are also several additional CheY homologs, however it lacks CheB, CheR, and CheZ (Pittman *et al*., 2001; Foynes *et al*., 2000). Chemotaxis mutants deficient in either its two CheY

homologs and a CheACheY2 mutant were impaired in their ability to colonize mice, gnotobiotic pigs, and gerbils thus demonstrating the importance of chemotaxis in establishing infection of *H. pylori* (Foynes *et al*., 2000; McGee *et al*., 2005).

Vibrio cholerae

In contrast, the human pathogen *Vibrio cholerae* appears to require motility but not chemotaxis to establish infection. *V. cholerae* inhabits rivers and other aquatic environments and has the ability to infect humans. It colonizes the small intestine and releases cholera toxin that results in severe diarrhea. This results in shedding large numbers of bacteria, a cycle leading to epidemic outbreaks of disease (Gill 1977).

V. cholerae possesses three che operons only one of which is involved in chemotaxis (Gosink *et al*., 2002). Counterintuitively, studies found that motility but not chemotaxis was required for establishing infection (Gardel and Mekalanos, 1996; Butler and Camilli, 2004). Moreover, smooth swimming motility, resulting from a CCW bias of flagellar rotation, results in higher rates of infection in the mouse compared to either wild type or cells with a CW bias presumably because these cells are able to spread further into the small intestine (Butler and Camilli, 2004). Interestingly, several studies demonstrate that chemotaxis genes are down-regulated in stool isolates from infected humans providing further evidence that motility but not chemotaxis is required for infection by *V. cholerae* (Merrel *et al*., 2002; Butler *et al*., 2006).

Campylobacter jejuni

Campylobacter jejuni, which has a single che operon, colonizes the gastrointestinal tract of many birds and other animals resulting in a commensal relationship, however this bacterium also infects humans and is a leading cause of bacterial gastroenteritis characterized by bloody diarrhea. Earlier studies demonstrated the importance of chemotaxis and motility in establishing infection of *C. jejuni* in mouse and ferret models for disease (Yao *et al*., 1997; Takata *et al*., 1992), but until recently their role in establishing a commensal relationship was unknown. A large scale genetic screen found that two specific MCPs and cheY are required for *C. jejuni* to establish commensalisms with a vertebrate host, the chick (Hendrixson and DiRita, 2004).

Vibrio fischeri

The bioluminescent marine bacterium *Vibrio fischeri* is able to colonize the light organ of the squid, *Euprymna scolopes.* This symbiotic relationship provides the nocturnal squid with antipredatory protection by eliminating shadows cast by moonlight and seen from below by potential predators. In exchange, the bacteria receive nutrients from the epithelial cells lining the light organ (Ruby, 1996; Ruby and McFall-Ngai, 1999; Visick and McFall-Ngai, 2000). Since motility is absolutely required for colonization (Graf *et al*., 1994), and *V. fisheri* exhibits chemotaxis towards a component of squid light-organ mucous, N-acetylneuraminic acid, it is likely that chemotaxis plays a role in establishing this symbiosis.

A database search (Ulrich and Zhulin, 2007) reveals that *V. fisheri* has one che cluster. Further experiments with genetically defined mutations of the chemotaxis

components in this cluster as well as *in vivo* experiments are required to more fully understand the role of chemotaxis in establishing the *V. fischeri* symbiosis with *E. scolopes.*

Chemotaxis and Plant-associated Bacteria

As with other host/microbe relationships, the types of relationships between soil bacteria and plants range from pathogenic to beneficial. Plant pathogenesis by a soil bacterium is exemplified by *Agrobacterium tumefaciens* while beneficial associations are represented by the genera *Rhizobium*, *Azospirillum,* and various pseudomonads (Vande Broek and Vanderleyden, 1995).

Agrobacterium tumefaciens

The soil bacterium *Agrobacterium tumefaciens* is the plant pathogen that causes crown gall tumor. Because of its ability to infect most dicotyledonous plants and transfer genetic material to the plant genome, *A. tumefaciens* is both an important model for plant pathogenesis and an indispensable tool for plant molecular geneticists (Sheng and Citovsky, 1996).

A. tumefaciens exhibits chemotaxis towards many different components of plant root exudates, including sugars, amino acids (Loake *et al*., 1988) and phenolics, known to be inducers of *A. tumefaciens* virulence genes (Shaw *et al*., 1991). However, studies are inconsistent as to whether chemotaxis and motility are required for infection and depend on the root growth substrate i.e. sand or soil and on whether inoculation of the roots is direct or indirect (Vande Broek and Vanderleyden 1995).

As stated above not all plant-bacteria interactions are disadvantageous for the plant—some are essential. Members of the Rhizobia can infect the roots of host plants and differentiate into cells called bacteroids that reside within root nodules. A single bacterium typically gives rise to all the cells within a nodule. The bacteroid cells fix nitrogen into ammonium which can be assimilated by the plant. In exchange the plant provides the bacteroids with nutrients. This process of biological nitrogen fixation is essential not only for the individual plant but also for all life on earth. It is estimated that 65% of nitrogen in the biosphere derives from biological nitrogen fixation. A majority of this comes from the rhizobium-legume symbiosis.

Sinorhizobium meliloti

S. meliloti is the rhizobial species whose chemotaxis system is best understood. Studies have revealed that chemotaxis in *S. meliloti* bears similarities to the *E. coli* paradigm, but it also differs in several respects. *S. meliloti* has two che operons, but chemotaxis is controlled by a single operon. The second could be involved in pili-based motility (Schmitt, 2002). There are relatively few chemoreceptors, nine compared to the five of *E. coli* and 27 of *R. leguminosarum* (Miller *et al*., 2007). *S. meliloti* has between five and ten peritrichous flagella per cell (Gotz, *et al*., 1982).

Both the flagellar structure and mechanism for changing swimming direction of *S. meliloti* is different from *E. coli*. *S. meliloti* flagella are rigid and complex. Like *E. coli* flagella they form a bundle that propels the cell forward, but because of their rigidity they cannot undergo the structural changes that occur when *E. coli* flagella are switched from CCW to CW rotation. Instead of switching direction, *S. meliloti* varies the rotational

speed of its unidirectional flagella to effect changes in swimming direction. Flagella form a bundle that propels the cell forward when they rotate unidirectionally at the same speed. Binding of CheY-P to the flagellar motor causes a decrease in the speed of rotation which in turn causes the flagellar bundle to disassociate and the cell is reoriented (Gotz and Schmitt, 1987).

This ability to vary the rotational speed of the flagella is not well understood, but requires at least two proteins not found in *E. coli*: MotC, and MotE (Platzer *et al*., 1997; Eggenhofer *et al*., 2004; Eggenhofer *et al*., 2006). MotA and MotB form the stator of the flagellar motor—the stationary torque generating part of the flagellum that surrounds the basal body (Blair and Berg, 1990; Stolz and Berg, 1991). MotC is a periplasmic protein that interacts with the periplasmic portion of MotB (Platzer *et al*., 1997). MotE is a chaperone protein specific for MotC that is responsible for its proper folding and stability in the periplasm (Eggenhofer *et al*., 2004).

In addition to its different motility mechanism, the *S. meliloti* chemotactic signaling pathway also deviates from the *E. coli* model. Like most α-proteobacteria, *S. meliloti* lacks a CheZ phosphatase. The *S. meliloti* che operon encodes two cheY homologs, $CheY_I$ and $CheY_{II}$, that differ enough in sequence that they are unlikely to be redundant (Greck *et al*., 1995). Mutational studies have revealed that $CheY_{II}$ is the major response regulator that interacts with the flagellar motor to slow its rotation while $CheY_I$ modulates $CheY_{II}$ activity by acting as a phosphate sink (Sourjik and Schmitt, 1996; Scharf and Schmitt, 2002).

Despite its well characterized chemotaxis system, further studies *in vivo* using molecularly characterized mutants are required to demonstrate the role of chemotaxis in establishing symbiosis with the *S. meliloti* host plant alfalfa.

Rhizobium leguminosarum

Rhizobium leguminosarum bv. *viciae*, a member of the alpha-proteobacteria, inhabits the soil and can live both planktonically or endosymbiotically within the roots of its leguminous host plant *Pisum sativa*, the common pea plant. This relationship is highly specific and its establishment is the result of a complex process that involves the exchange of numerous chemical signals between the bacterium and the plant. This results in infection of the plant root by individual bacterial cells which then grow into elongated cells called infection threads. The bacteria undergo a cellular differentiation process resulting in cells called bacteroids that reside within the roots in specialized structures called nodules. The bacteroids within each nodule are usually of clonal origin. The bacteroids within each nodule fix atmospheric nitrogen into ammonium which can be used by the plant. In exchange the plant provides the bacteria with nutrients.

Chemotaxis has long been thought to play an important role in establishing this endosymbiotic relationship. Several studies have investigated chemotaxis in *Rhizobium leguminosarum* bv. *viciae* and its role in establishing symbiosis with the pea plant. Early studies by Bowra and Dilworth (1981) and Gaworzewska and Carlile (1982) sought to identify both the optimum conditions for studying chemotaxis in *R. leguminosarum* and determine which compounds were strong chemoattractants using the capillary assay. These two studies identified some 37 chemoattractants for *R. leguminosarum* including

sugars, sugar alcohols, amino acids, and organic acids. A separate study by Armitage *et al*. (1988) demonstrated chemotaxis towards the flavonoid compounds apigenin and naringenin which are produced by the pea plant and induce transcription of nodulation genes in *R. leguminosarum*.

Studies by Hynes and colleagues identified two MCPs that may play a role in competitive nodulation, MCPB and MCPC (Yost *et al*., 1998). This group also demonstrated that components of the chemotaxis system are downregulated in bacteroids (Yost *et al*., 2004).

Despite these studies, motility and the chemotaxis system of *R. leguminosarum* remained largely uncharacterized. The major objective of this thesis is to characterize the motility and chemotaxis system of *R. leguminosarum* and determine the contribution of chemotaxis in establishing symbiosis with the host plant using genetically defined mutants (Chapter 2). We found that of the two chemotaxis gene clusters encoded by *R. leguminosarum*, *che1* is the dominant che cluster responsible for chemotaxis and is essential for competitive nodulation (Miller *et al*., 2007).

Because *che1* is the major che cluster and is required for competitive nodulation we next turn our attention to the chemotaxis transducer it encodes, IcpA-*Rl* (Chapter 3). We present evidence that IcpA-Rl is an energy taxis transducer and represents a novel function of the globin coupled sensor domain in sensing energy.

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CHAPTER 2

THE MAJOR CHEMOTAXIS GENE CLUSTER OF *RHIZOBIUM LEGUMINOSARUM* BV. *VICIAE* IS ESSENTIAL FOR COMPETITIVE NODULATION.

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A published manuscript: Miller, L.D., Yost, C.K., Hynes, M.F., and Alexandre, G. (2007) The major chemotaxis gene cluster of *Rhizobium leguminosarum* bv. *viciae* is essential for competitive nodulation. *Mol Microbiol* **63**: 348—362.

Key words: Chemotaxis, Rhizobium leguminosarum, Nodulation, Motility

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SUMMARY

Rhizobium leguminosarum bv. *viciae* strain 3841 is a motile alpha-proteobacterium that can establish a nitrogen-fixing symbiosis within the roots of pea plants (*Pisum sativum*) and other hosts. In order to determine the contribution of chemotaxis to the lifestyle of *R. leguminosarum*, we have characterized the function of two chemotaxis gene clusters (*che1* and *che2*) in controlling motility behaviour. We have found that both chemotaxis gene clusters modulate the motility swimming bias of *R. leguminosarum* cells and that the *che1* cluster is the major pathway controlling swimming bias and chemotaxis. The *che2* cluster also contributes to swimming bias, but has a minor, if any, effect on chemotaxis. Using competitive nodulation assays, we have also demonstrated that a functional *che1* cluster, but not the *che2* cluster, promotes competitive nodulation of pea*s*. This finding implies that the environmental cue(s) triggering chemotaxis of *R. leguminosarum* bv. *viciae* cells toward the roots of pea and facilitating colonization are likely to be processed through the *che1* cluster despite the contribution of both *che* clusters to swimming behavior. A phylogenetic analysis of the distribution of *che1* and *che2* orthologs in the alpha-proteobacteria together with our results allow us to propose

that *che1* homologs are major controllers of chemotaxis and host association in the Rhizobiales.

INTRODUCTION

Motility and chemotaxis provide bacteria with increased fitness by allowing them to locate and navigate toward niches that best support their growth. In many environments, such as the soil and rhizosphere, where steep concentration gradients of nutrients exist, bacterial chemotaxis is thought to play a critical role in structuring microbial communities. Similarly, locating the host or the appropriate tissue may also promote various host-microbe relationships (Vande Broek and Vanderleyden, 1995; Alexandre *et al*., 2004).

Rhizobia are motile alpha-proteobacteria that can establish a nitrogen-fixing symbiosis within the roots of leguminous plants. The formation of root nodules, in which bacterial nitrogen fixation occurs, results from the highly specific interaction between rhizobia and their plant hosts (Oke and Long 1999). In the *Rhizobium*-legume association, the site of entry for the *Rhizobium* is the tip of a developing root hair, where root exudates are abundant (Van Rhijn and Vanderleyden, 1995). Bacterial chemotaxis toward chemoattractants originating from the roots of the legume host may promote infection and subsequent nodulation by directing the rhizobia to the proper infection site. In agreement with this hypothesis, it has been previously shown that different species of rhizobia, including *Rhizobium leguminosarum* and *Sinorhizobium meliloti*, displayed chemotaxis towards root exudates in various *in vitro* assays (Aguilar *et al*., 1988;

Armitage *et al*., 1988; Caetano-Anolles *et al*., 1988a; Barbour *et al*., 1991; Kape *et al*., 1991; Dharmatilake and Bauer, 1992). Furthermore, two different mutants of *Rhizobium leguminosarum* bv. *viciae* each impaired in a single MCP-encoding gene were less competitive in pea root nodulation than the parental strain (Yost *et al*., 1998). Although results from these studies support the notion that bacterial chemotaxis contributes to the rhizobia-legume association, they do not analyze the complete set of chemotaxis proteins and therefore provide a partial perspective on the exact role that chemotaxis plays in the bacterium-plant association. Although the molecular mechanism of chemotaxis in *S. meliloti* is arguably one of the best described in the alpha-proteobacteria (Greck *et al*., 1995; Sourjik and Schmitt, 1996; Schmitt, 2002; Attmanspacher *et al*., 2005), the contribution of this behavior to the establishment of a specific association with its legume host has not been determined using genetically defined bacterial mutants.

Bacteria sense environmental stimuli and relay this signal to the flagellar motor via a signal transduction pathway that ultimately controls the direction (or speed) of flagellar rotation (Wadhams and Armitage, 2004). The conserved chemotaxis signal transduction system is comprised of membrane-bound chemotaxis transducers (so called methyl-accepting chemotaxis proteins or MCPs), coupled to a cytoplasmic histidine kinase CheA via the CheW protein. Upon chemostimulation, CheA becomes autophosporylated at a conserved histidine residue and then transfers its phosphate to its cognate response regulator, the CheY protein. Phospho-CheY binds to the flagellar motor, an event that ultimately leads to a switch in the direction of flagellar rotation (or a variation in the rotary speed of the flagellar motor). The CheB methylesterase is activated by phosphotransfer from CheA and balances the activity of the

methyltransferase CheR protein that constitutively adds methyl groups to the C-terminal domains of MCPs (Armitage, 1999). Additional chemotaxis proteins may also be found in different bacterial species and variation on the theme is the rule rather than the exception (Szurmant and Ordal, 2004).

Sensing specific chemical(s) released in the environment such as the root exudates is a likely requirement in the establishment of the *R. leguminosarum*-pea plant association. However, the molecular mechanisms of chemotaxis in this organism remained to be determined. The *Rhizobium leguminosarum* biovar *viciae* strain 3841 genome was recently sequenced (Young *et al*., 2006). In order to determine the contribution of chemotaxis to the biology of *R. leguminosarum,* we have searched the genome of *R. leguminosarum* for homologs of chemotaxis proteins and found two gene clusters, which we termed *che1* and *che2*. In the present report, we describe the contribution of *che1* and *che2* to chemotaxis in *R. leguminosarum* bv. *viciae* and their role in competitive nodule formation.

RESULTS

Rhizobium leguminosarum *biovar* viciae *are motile by one to two sub-polar flagella*

When we initiated this study, little information was available regarding the motility of *R. leguminosarum* bv. *viciae* except the apparent requirement of divalent cations such as Ca^{2+} for synthesis or assembly of the flagellum and efficient motility (Smit *et al*., 1989). We have tested motility of *R. leguminosarum* strain 3841 in various buffers and found that a greater number of cells were motile in the chemotaxis buffer

supplemented with Ca^{2+} compared to that lacking Ca^{2+}, in which cells tend to lose motility rapidly. We have used transmission electron microscopy to determine the number and arrangement of flagella in *R. leguminosarum* bv. *viciae* strain 3841 and found that most cells possess one to two sub-polar flagella (Figure 2.1A). When observed under the bright field microscope, cells of *R. leguminosarum* swam with a three-dimensional walk consisting of straight runs punctuated by changes in swimming direction (Figure 2.1B). Analysis of swimming paths of free-swimming cells tracked with computerized motion analysis software shows that the paths tend to be curved with instances of changes in direction characterized by either small loops or broad angles. The observation of the rotation of motile cells tethered by their flagella suggested that *R. leguminosarum* cells swim by rotating unidirectional flagellar motors (Figure 2.1C). Furthermore, the speed of flagellar rotation appeared to vary during the time of the observation (Figure 2.1C). This analysis also suggests that the sharp changes in the swimming direction and reorientation of the cells in a new swimming direction may be brought about by abrupt changes in the flagellar rotational speed (Figure 2.1C). We could not detect instances of pauses or stops in tethered cells using this approach but only changes in rotary speed. Therefore, we hypothesize that swimming cells of *R. leguminosarum* bv. *viciae* modulate the rotary speed of the flagellar motor in order to re-orient randomly in a new swimming direction. Cells grown with mannitol as the carbon source swam with an average speed of about 38.3 ± 6.7 μm sec^{-1} with changes in the swimming direction occurring about every 3 seconds. Varying the carbon source did not have a measurable effect on swimming speed or the probability of change in swimming direction (data not shown).

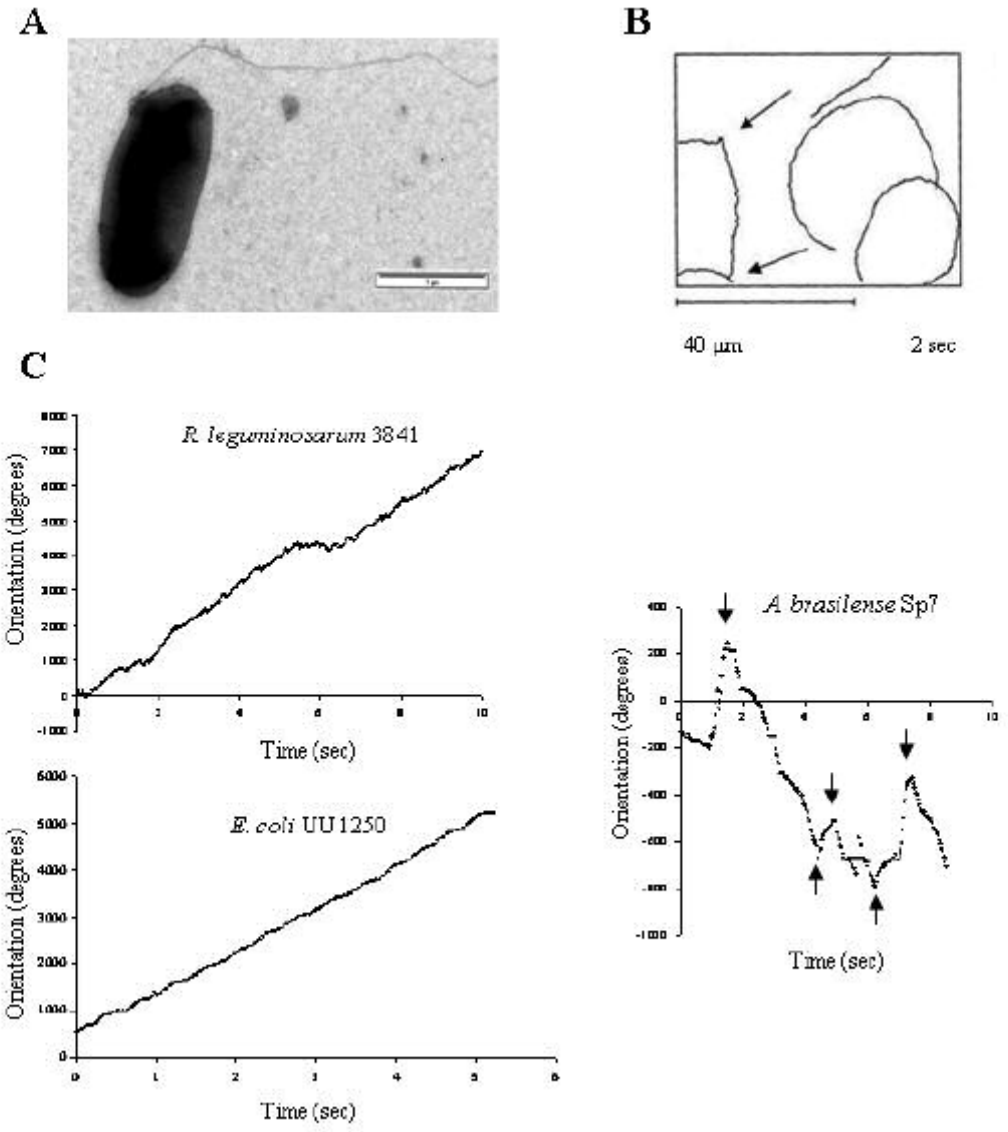

Figure 2.1 Motility of cells of *R. leguminosarum* bv. *viciae*. A) A transmission electron micrograph of *R. leguminosarum* bv. *viciae* showing a single sub-polar flagellum. Cells were negatively stained with 1% uranyl acetate. Bar, 1 µm. B) Examples of swimming paths of *R. leguminosarum* bv. *viciae* wild type strain grown in minimal medium with pyruvate (10 mM) as determined using the Hobson BacTracker. Arrows indicate incidents of abrupt changes in swimming direction. The cells were tracked for 2 s. C) Tethered cells were analyzed to determine the rotational bias of *R. leguminosarum* bv. *viciae* flagella. The direction of orientation of individual cells is measured in degrees and given as a function of time in seconds. As cells rotate in one direction, the orientation value constantly increases linearly. Rotation in the opposite direction causes the

Figure 2.1 (continued) orientation value to decrease. This accounts for the variation between graphs in y-axis scale. Results for individual representative cells are shown in each graph. Representative tethered cells of *R. leguminosarum* appear to rotate in one direction at a variable speed. A Representative tethered cell of *A. brasilense* can reverse the rotational direction of the single polar flagellum (Zhulin and Armitage, 1993) and is shown for comparison. Arrows indicate instances of reversal of the direction of flagellar rotation. *E. coli* UU1250 is a mutant strain lacking all chemoreceptors (Ames *et al.,* 2002). *E. coli* UU1250 cannot reverse the direction of flagellar rotation and is shown for comparison.

The genome of Rhizobium leguminosarum *biovar* viciae *strain 3841 encodes two chemotaxis gene clusters*

Two clusters of genes encoding homologs of known chemotaxis proteins, which we termed *che1* and *che2*, were found and predicted to be located on the chromosome (Figure 2.2). Chemotaxis genes identified on the *R. leguminosarum* chromosome in each gene cluster, are transcribed in a single direction and genes in this cluster are separated by 64 bp (between *mcp* and first orf) or less. In addition, the *cheY*, *cheA*, and *cheW* genes from the *che2* cluster appear to be translationally coupled. Furthermore, genes located upstream and downstream of the *che2* cluster are transcribed in opposite directions. Putative ribosome binding sites were predicted upstream of the *mcp* gene homolog and the orf in the *che1* cluster and upstream of the $cheY_{III}$ gene in the *che2* cluster, suggesting that each cluster may comprise an operon (Lukashin and Borodovsky, 1998), similar to the organization of homologous chemotaxis operons in other bacteria and archaea (Hauwaerts *et al*., 2002; Szurmant and Ordal, 2004). Both chemotaxis clusters encode the conserved set of CheAWYRB homologs found in homologous chemotaxis operons from *Escherichia coli* and *Bacillus subtilis* (Armitage, 1999; Bren and Eisenbach, 2000; Szurmant and Ordal, 2004). Interestingly, the chemotaxis cluster *che1*is orthologous to the well-studied chemotaxis operon controlling flagellar motility in *S. meliloti* (Greck *et al*., 1995; Sourjik and Schmitt, 1996), and *che2* is orthologous to genes coding for one of the chemotaxis pathways involved in controlling flagellar motility in *Rhodobacter sphaeroides* (Hamblin *et al*., 1997a), which is also a well-studied model. Noticeably, the *che1* and *che2* clusters from *R. leguminosarum* bv, *viciae* have essentially identical gene order with corresponding orthologous clusters in *S. meliloti* and *R. sphaeroides*,

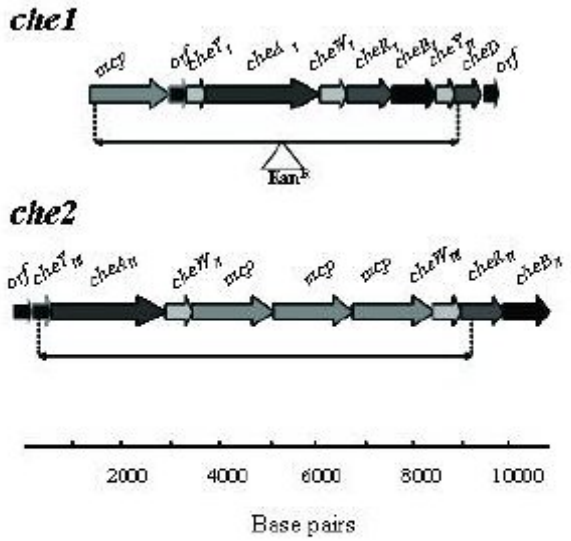

Figure 2.2 Schematic diagram showing the genetic organization of the *R. leguminosarum* bv. *viciae* strain 3841 chemotaxis gene clusters *che1* and *che2*. The double arrow-headed line below each operon indicates the region deleted in each of the *che* mutants. A kanamycin resistance cassette was inserted into the mutants lacking *che1* as shown. A scale of the DNA region spanning each chemotaxis gene cluster is shown below for reference.

respectively, that have each been shown to comprise operons. The conservation of gene order and the short gaps between genes within the clusters are two major features of bacterial operons (Yan and Moult, 2006). Altogether, this strongly suggests an operon organization for *che1* and *che2* in *R. leguminosarum* bv. viciae. The *che1* cluster is also predicted to encode two homologs of the CheY response regulator, an MCP homolog, a CheD homolog, and a conserved hypothetical protein of unknown function (orf). The *che2* cluster is predicted to encode two CheW homologs and three MCP homologs (Figure 2.2). We did not find any genes potentially encoding the CheZ phosphatase in the complete genome sequence, consistent with previous observations that CheZ homologs are not present in alpha-proteobacteria (Szurmant and Ordal, 2004). In addition to the two chemotaxis clusters, two genes apparently encoding CheW homologs, present at separate loci on the chromosome, and a total of 27 genes coding for MCP homologs are present on the chromosome and four of the six plasmids of *R. leguminosarum* 3841. The plasmid location of several *mcp* genes has been previously reported (Brito *et al.*, 1996; Yost *et al*., 1998; Yost *et al.*, 2003) in various strains of *R. leguminosarum*. The function of the MCPs encoded in the *che1* and *che2* clusters is unknown and none of the genes coding for MCPs that were previously mutated and analyzed (Brito *et al.*, 1996; Yost *et al*., 1998; Yost *et al*. 2003) are located in the *che1* or the *che2* clusters.

We have constructed transcriptional fusions of putative promoter regions located upstream of the *che1* and *che2* clusters with a promoterless *gusA* gene and measured the activity of the putative promoter under different growth conditions as described in *Experimental Procedures*. We found that both transcriptional fusions were significantly

expressed under all growth conditions tested, relative to a control (Table 2.1), suggesting that the corresponding *che* clusters are constitutively expressed in free-living cells.

In order to determine the contribution of each gene cluster to the *R. leguminosarum* chemotactic response, we generated deletion mutants using an allelic exchange suicide vector incorporating the *sacB* gene for positive selection as described previously for *R. leguminosarum* (Quandt and Hynes, 1993). We constructed a deletion-insertion mutant in *che1* by deleting the DNA region spanning the C-terminal region of *mcp* to the *cheD* gene and inserted a kanamycin resistance cassette (Figure 2.2). We constructed a markerless mutant in *che2* by deletion of the DNA coding sequences between $cheY_{III}$ and $cheR_{II}$. The remaining portion of the *cheR* gene (approximately half) is unlikely to code for a stable, functional protein and is missing the active site. A mutant lacking both chemotaxis operons, Δ*che1,2*, was constructed by using the deletion construct for *che1* to delete this operon from Δ*che2*. The mutations did not have any effect on the growth rate and doubling time on various carbon sources (pyruvate, galactose, mannitol, TY medium), and all three mutants were motile.

Che1 is the major chemotaxis cluster that controls chemotaxis in a spatial gradient assay

To determine the role of each *che* cluster in chemotaxis, we compared the parent strain of *R. leguminosarum* and the chemotaxis mutants using the spatial gradient assay (swarm plates). A variety of growth substrates that are typical of plant root exudates previously identified as attractants for *R. leguminosarum* (Bowra and Dilworth, 1981; Gaworzewska and Carlisle, 1982; Yost *et al*., 1998), were tested (for example, Figure 2.3A, 2.3B). There was no obvious difference in doubling time between any of the

Table 2.1. Expression of *che1* and *che2* determined by *gusA* reporter fusions.

	β-glucuronidase activity (nmol p-nitrophenol min^{-1} OD_{600}^{-1})[a]		
Growth media	control	*che*1 promoter	*che*2 promoter
TY	2.44 ± 0.147	34.1 ± 11.1	19.1 ± 2.24
Mannitol	3.27 ± 0.504	56.5 ± 5.04	29.2 ± 7.56
Galactose	2.79 ± 1.04	66.0 ± 6.11	30.0 ± 7.39

[a] Values represent the mean and standard deviation of four replicates from two independent cultures. Levels of reporter gene expression were significantly different between the *che1* and *che2* promoter constructs and the promoterless control as determined by a Student's *t*-test ($P < 0.05$).

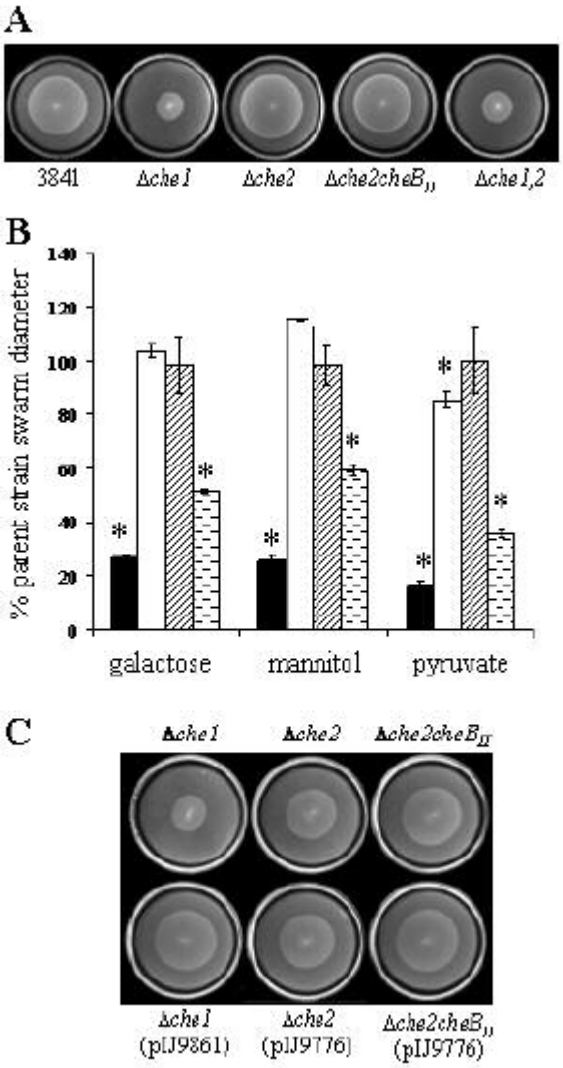

Figure 2.3 Chemotaxis of *R. leguminosarum* and the Δ*che* mutants to oxidizable substrates. A) Spatial gradient assay in minimal medium with galactose 1mM as the chemoeffector. Plates were inoculated with the same number of cells and incubated for 5 days and demonstrate that all strains were capable of producing rings under these conditions. B) Quantitative spatial gradient assay (swarm plate assay): carbon sources were tested at a final concentration of 1 mM. The diameter of swarm rings formed by Δ*che1* (black bars), Δ*che2* (white bars), Δ*che2cheB$_{II}$* (hatched bars) and Δ*che1,2* (dashed bars) mutants are expressed in per cent relative to the parent strain (taken as 100%). Similar results were obtained with glutamate, maltose, proline and succinate tested as chemoeffectors. Error bars represent the standard deviation from at least four repetitions. An asterisk (*) indicates a statistically significant difference between the mutant and parent strain as determined by a Student's *t*-test (alpha level, $P < 0.05$). C) Complementation of the chemotaxis defect of the *che* mutants with cosmids carrying

Figure 2.3 (continued) parental genes for each of the *che* clusters in a spatial gradient assay with pyruvate 1 mM as the chemoeffector. Similar results were obtained with mannitol (1 mM), galactose (1 mM), and succinate (1 mM).

strains used on three of the compounds, mannitol, galactose and pyruvate. Therefore, any difference in swarm diameter can be attributed to a difference in either swimming bias, chemotactic ability, or both. We found that on all chemoeffectors tested the parent strain and all three mutants formed swarms without a distinct outside ring typical of many other chemotactic species (Figure 2.3A). Similar swarms were observed for the parent strain and the mutants in plates lacking a carbon source (data not shown) or in plates solidified with high purity agar (Noble agar) or agarose indicating that this was not due to a response to a contaminant in the agar, but just a result of random translocation of motile cells through the matrix. The relative swarm diameter was the only consistent difference between the parent strain and the mutants in this assay. Similar observations were made by Sourjik *et al*. (2000) with closely related *S. meliloti*, where the swarm diameter was the only parameter distinguishing chemotactic versus non-chemotactic strains. The Δ*che1* and Δ*che1,2* mutants formed swarms of a significantly smaller diameter relative to the parent strain regardless of the chemoeffector tested. The swarm diameter of the Δ*che2* mutant relative to the parent strain was dependent on the chemoeffector tested (Figure 2.3B). The Δ*che2* mutant formed swarms similar to that of the parent on some (galactose, mannitol) but not all (pyruvate) chemoeffectors tested. Complementation of the Δ*che1* and Δ*che2* mutants with cosmids pIJ9861 and pIJ9776 that carry parental genes for the *che1* and *che2* clusters, respectively, restored swarming behavior (Figure 2.3C). Since a Δ*che1* mutant and a Δ*che1,2* mutant are both significantly impaired in chemotaxis to all chemoeffectors, whereas the *che2* cluster has only minor defects, we conclude that the *che1* cluster controls most chemotactic responses in *R. leguminosarum*.

Che1 controls the swimming motility bias while che2 modulates it via $CheB_{II}$.

The differences in the swarm diameters between the parent and the mutants could be due to differences in the ability to detect chemical gradients and/or different swimming biases. We determined the motility bias by measuring the reorientation frequency, defined as the number of changes in the swimming direction per second (Hyakutake *et al*., 2005), for free-swimming cells of the parent and the chemotaxis mutants on the same chemoeffectors as those tested in the spatial gradient assay (Table 2.2). The Δ*che1* mutant swam with a low reorientation frequency relative to the parent strain, which is equivalent to a smooth swimming bias. In contrast, the Δ*che2* mutant exhibited a swimming pattern with frequent changes in the swimming direction, regardless of the substrates used for growth. The Δ*che1,2* mutant had a steady-state swimming bias similar to that of the Δ*che1* mutant under all conditions tested. The swimming motility biases of the Δ*che1* and the Δ*che2* mutants were complemented by expression *in trans* of the parental *che1* and *che2 clusters* from cosmids pIJ9861 and pIJ9776, respectively (Figure 2.4*).* These results indicated that both *che* clusters may have a role in controlling the motility bias in *R. leguminosarum* bv. *viciae.* Comparison of the swimming biases of the Δ*che1,* Δ*che2* and the Δ*che1,2* mutants further suggests that the signaling output from the *che1* cluster is a major controller of the swimming bias in *R. leguminosarum* since a *Δche1* and a *Δche1,2* mutant have a similar swimming bias. This analysis also indicates that the *che2* cluster probably functions to decrease the probability of reorientation frequency.

The $cheB_{II}$ gene is not deleted in the markerless Δ*che2* mutant. We tested the hypothesis that the motility bias of the *Δche2* mutant resulted from the presence of an

intact *cheB$_{II}$* gene by constructing an insertion mutant in the *cheB$_{II}$* gene in the Δ*che2* mutant background, yielding a Δ*che2cheB$_{II}$* mutant. We characterized the motility phenotype of this mutant using the same assays as for the *Δche1*, *Δche2* and *Δche1,2* mutants (Figure 2.3; Table 2.2). We found that mutating *cheB$_{II}$* in the Δ*che2* mutant background restored the parent swarm diameter in the swarm plate assay (Figure 2.3A, B). The most dramatic effect of mutating the *cheB$_{II}$* gene in the *Δche2* mutant background was seen in the steady-state motility bias of free-swimming cells (Table 2.3). Mutating *cheB$_{II}$* suppressed the high reorientation frequency observed in the *Δche2* mutant on all chemoeffectors tested: a Δ*che2cheB$_{II}$* mutant had essentially the same steady-state swimming motility bias (reorientation frequency) as the parent strain. This suggests that the motility bias of the Δ*che2* mutant is due to the presence of an intact *cheB$_{II}$* gene in this mutant. Expressing the *che2* cluster from pIJ9776 in the Δ*che2cheB$_{II}$* mutant did not affect the reorientation frequency or swarming behavior (Figure 2.3C; Figure 2.4). This suggests that the motility bias of the Δ*che2* mutant may be due to the presence of an intact *cheB$_{II}$* gene in this mutant.

Only the che1 cluster is essential for competitive nodule formation

In control experiments, the Δ*che1* or Δ*che2* or Δ*che2cheB$_{II}$* mutants when inoculated alone onto pea plants, formed functional nodules, with no difference in the number and morphology from roots with parental nodules. In addition, inoculated plants did not show any sign of nitrogen starvation as compared to uninoculated control plants and were otherwise indistinguishable from plants inoculated with the parental strain, including amount of dry matter produced per plant. We tested the ability of each of the Δ*che*

Table 2.2. Reorientation frequency of *R. leguminosarum* and *che* mutant free-swimming cells (sec^{-1}).

carbon source [a]	Parent	Δ*che1*	Δ*che2*	Δ*che1,2*	Δ*che2che*B_{II}
Galactose	0.34 ± 0.01	0.05 ±0.07[b]	0.46 ± 0.05[b]	0	0.24 ± 0.09
Mannitol	0.30 ± 0.01	0.03 ± 0.01[b]	0.38 ± 0.02[b]	0.02 ± 0.02[b]	0.36 ± 0.09
Pyruvate	0.22 ± 0.01	0.02 ± 0.01[b]	0.40 ± 0.06[b]	0.02 ± 0.02[b]	0.19 ± 0.04

[a] Cells were grown in minimal media with the carbon sources indicated at 10 mM (final concentration) to mid-exponential growth phase. The swimming behavior of cells was video-recorded and the reorientation frequency was measured as described in *Experimental Procedures*.

[b] Values for reorientation frequency were significantly different between the parent strain and che mutants as determined by a Student's *t*-test ($P < 0.05$).

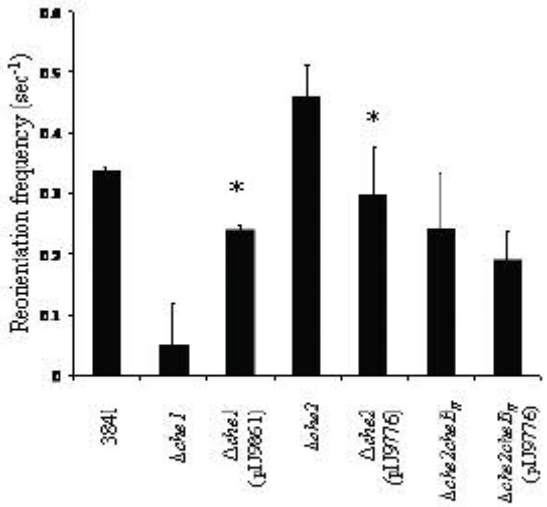

Figure 2.4 The parental motility bias is restored in complemented Δ*che1* and Δ*che2* mutants. The reorientation frequency was measured on free-swimming cells grown in minimal medium with galactose 10 mM. Cosmids that complement the *che* mutants were able to restore the decreased reorientation frequency in the case of the Δ*che1* mutant and reduce the increased reorientation frequency in the case of the Δ*che2* mutant. The reorientation frequency of Δ*che2cheB*$_{II}$ (pIJ9776) was not statistically significantly different from Δ*che2cheB*$_{II}$. Error bars represent standard deviations from the means of 30 cells each from three independent cultures. An asterisk (*) indicates a statistically significant difference between the cosmid bearing strains and the corresponding *che* mutant strains based on a Student's *t*-test ($P < 0.05$).

mutants of *R. leguminosarum* to form nodules on pea plant (*Pisum sativum*) roots in competitive nodulation experiments with the parent strain. The results shown in figure 2.5 demonstrate that the Δ*che1* mutant was unable to compete with parent strain *R. leguminosarum* 3841 for nodulation of pea plants even when the Δ*che1* mutant to parent inoculum ratio was increased to 10:1. When a cosmid encoding parental genes for the *che1* cluster (pIJ9861) was introduced into the Δ*che1* mutant, it restored the ability to compete with the parent strain for nodulation. In contrast, we found that neither the Δ*che*2 nor the Δ*che2 cheB*$_{II}$ mutant was affected in the ability to form nodules on pea roots in competition with the parental strain. The Δ*che1*/Δ*che*2 double mutant was also tested in competition assays, and, as expected, showed the same competition deficiency as the Δ*che1* single mutant. This deficiency was complemented by the presence of pIJ9861 (data not shown). Together, these results show that the *che1* chemotaxis cluster is essential for promoting competitive nodule formation while the *che2* cluster is dispensable.

Distribution of the che1 and che2 clusters in rhizobial species.

We have searched completely sequenced genomes of other alpha-proteobacterial species for homologs of the *che1* and *che2* clusters (see *Experimental Procedures*) and found that both clusters are present in other alpha-proteobacteria, including members of the Rhizobiales (Figure 2.6). The *che1* and *che2* clusters are not always present together, except in the phylogenetically closely related *R. etli*. The *che1* cluster is orthologous to that found as the only chemotaxis operon in *A. tumefaciens* (Wright *et al*., 1998) and the only chemotaxis operon controlling flagellar motility in *S. meliloti* (Greck *et al*., 1995;

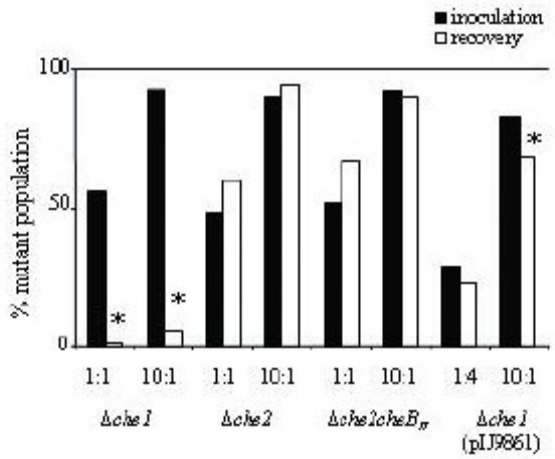

Figure 2.5 Nodulation competition between the Δ*che1*, Δ*che2* and Δ*che2cheB*$_{II}$ mutants and the parent strain of *R. leguminosarum* bv. *viciae* strain 3841. The ratios on the x-axis are expressed as Δ*che* mutant to parent strain 3841. The Δ*che1* mutant was rarely recovered from the harvested nodules. A 10 fold excess of the Δ*che1* mutant to parent could not restore its ability to successfully compete for nodulation sites. The Δ*che2* and Δ*che2cheB*$_{II}$ mutant were not impaired in the ability to compete against parent for nodulation sites regardless of the inoculation ratios. Complementation of the Δ*che1* mutant with pIJ9861 restored its ability to compete with the parent strain. Although the Δ*che1* (pIJ9861) strain is recovered at a slightly but significantly lower proportion when inoculated at the 10:1 ratio, the fraction of the complemented mutant recovered from nodules is still significantly higher compared with the competition between parent and the Δ*che1* mutant. Such difference was not observed at lower ratios (1:4). This indicates that competitiveness is restored. Two additional trials yielded similar results. An asterisk (*) indicates statistically significant (*P*-value < 0.001) differences between the inoculation ratio and recovery ratio, in a chi-square test.

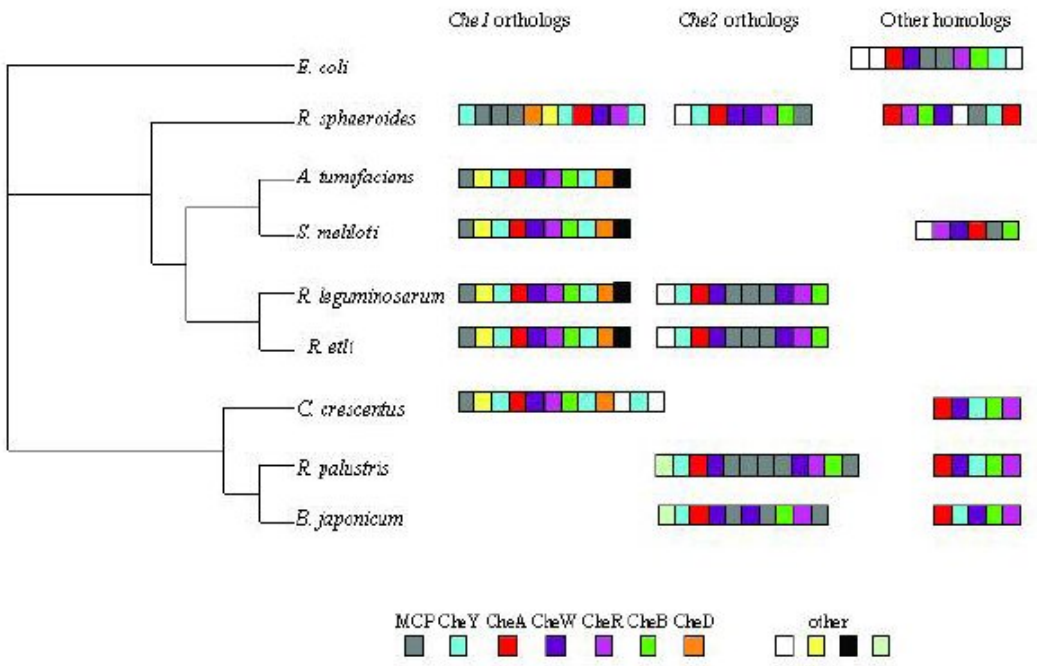

Figure 2.6 Distribution and organization of *che1* and *che2* gene cluster homologs in alpha-proteobacteria. Orthology relationships were determined as described in *Experimental procedures*. Other homologs refer to other chemotaxis-like operons in a given species and are listed for reference. These other *che* homologs are not necessarily phylogenetically or functionally related. The phylogenetic tree of the selected microorganisms is based on an alignment of 16S rRNA sequences, as described in *Experimental procedures*. The chemotaxis operon of *E. coli* was included for comparison.

Sourjik and Schmitt, 1996) and *Caulobacter crescentus* (Alley *et al*., 1991; Alley *et al*., 1992). *S. meliloti* has an additional che-like operon that may be involved in pilus based motility (Schmitt, 2002), and *C. crescentus* has an additional *che* operon whose function is unknown (Nierman *et al*., 2001). Although a *che1* ortholog is present in *R. sphaeroides*, it is not involved in controlling chemotaxis under laboratory conditions and its exact function is unknown (Ward *et al*., 1995). In contrast, it is the *R. leguminosarum che2* cluster that is orthologous to one of the two chemotaxis pathways involved in controlling flagellar motility in *R. sphaeroides* (Hamblin *et al*., 1997; Martin *et al*., 2001a,b). This analysis clearly illustrates that the function of chemotaxis clusters cannot easily be predicted by homology comparisons alone, even in phylogenetically close microorganisms. This comparison also suggests that *che1* orthologs are major pathways for controlling chemotaxis and plant association in the Rhizobiales.

DISCUSSION

The successful association between microorganisms and the respective hosts is dependent on the ability of the bacterial partner to detect the host presence followed by active colonization. The outcome of the association (pathogenic or beneficial) is then dependent on specific traits of the partners involved. Detection of the host legume by soil rhizobia and the active movement towards the roots by chemotaxis is thought to be important in the initial steps leading to the rhizobia-legume symbiosis. Previous experimental data obtained in *R. leguminosarum* bv. *viciae* (Bowra and Dilworth, 1981; Gaworzewska and Carlisle, 1982; Armitage *et al*., 1988; Yost *et al*., 1998; Yost *et al.,* 2004) suggested that bacterial chemotaxis and the ability to detect specific chemical

gradients originating from the host plant (via dedicated MCPs) is essential for the initiation of the host-microbe association but not its persistence, consistent with the long-standing hypothesis that chemotaxis enhances bacterial competitiveness in the rhizosphere (Brencic and Winans, 2005). The *R. leguminosarum* genome encodes two chemotaxis gene clusters each comprising a complete set of conserved chemosensory proteins which are homologous to chemotaxis operons controlling motility in closely related alpha-proteobacteria. The availability of the complete genome sequence provided us with the opportunity to establish which genes are essential for chemotaxis in this species and how this function influenced the association with the host plant.

Our data clearly demonstrate that the signaling output from the *che1* cluster controls the swimming motility bias and chemotaxis in *R. leguminosarum* bv. *viciae* strain 3841. Our analysis of the swimming behavior of the *Δche1* and *Δche1,2* mutants indicates that the default swimming bias is smooth swimming in *R. leguminosarum* (low probability of changes in the swimming direction) similarly to most other motile chemotactic bacteria, with the notable exception of *B. subtilis* (Armitage, 1999; Szurmant and Ordal, 2004). Remarkably, mutants lacking either of the two chemotaxis gene clusters had opposite motility biases in *R. leguminosarum* bv. *viciae*. However, we found that the contribution of the *che2* cluster to the motility bias was dependent on the presence of a functional *cheB$_{II}$* gene. These results indicate that Che2 may function to modulate the swimming bias and chemotaxis (via a contribution of CheB$_{II}$) but this is most likely not its primary (or only) function. A Δ*che2cheB$_{II}$* mutant had a chemotaxis phenotype and motility bias indistinguishable from that of the parent strain, further suggesting that (i) the signaling output from the *che2* cluster does not directly interact

with the rotational bias of the flagella, at least under the conditions tested in the present study; and (ii) the effect of $CheB_{II}$ on the motility bias is indirect.

Our results conclusively demonstrate that motile *R. leguminosarum* bv. *viciae* must be chemotactic in order to compete efficiently for nodulation. Once established in the nodules and differentiated into bacteroids, the competitive advantage provided by chemotaxis to free-living cells is no longer required, an assumption consistent with the observation that flagellar and chemotaxis genes are donwnregulated in nodules (Yost *et al.*, 2004, Becker *et al*., 2004).

In the absence of a functional *che1* cluster, cells are motile but they can no longer change the swimming direction, and thus they are unable to navigate in chemical gradients, including gradients created in the swarm plate assay or existing in the exudates of pea plants. We attribute the defect in nodulation competitiveness of the Δ*che1* mutant to its lack of chemotaxis *per se*, rather than an effect of the swimming bias as was shown in the case of the increased infectivity of non-chemotactic mutants of *Vibrio cholerae* (Butler and Camilli, 2004). First, we have compared three chemotaxis mutants with different motility biases (smooth swimming in Δ*che1*, high probability of changes in swimming direction in Δ*che2* and random swimming bias similar to that of the parent in Δ*che2cheB*$_{II}$) and different chemotaxis defects (Δ*che1* is null for chemotaxis, Δ*che2* has at best a moderate defect, and Δ*che2 cheB*$_{II}$ is not affected) in the competitive nodulation assay. Using these mutants, we have found that only the Δ*che1* mutant was impaired in nodulation competition. Second, a *mcpB* mutant previously found to be impaired in chemotaxis and nodulation competition with the parental strain (Yost *et al*., 1998) has a random swimming bias similar to the parental strain (Table 2.3). Taken together these

Table 2.3. Motile behavior and nodulation competition phenotype of parental *R. leguminosarum* VF39SM and the *mcpB* mutant.

strain	chemotaxis[a]	Competitive nodulation[a]	Motility bias[b]
Parent	+	+	0.36 ± 0.06
mcpB	-	-	0.37 ± 0.09

[a] Phenotypes described by Yost *et al*., 1998.

[b] The motility bias (reorientation frequency) was measured on cells grown in minimal media with mannitol 10 mM as described in *Experimental Procedures*.

data indicate that the lack of chemotaxis and not a particular steady-state swimming bias is responsible for the impaired competitiveness of the *mcpB* and the Δ*che1* mutants in nodulation of pea plants.

Many bacteria possess multiple chemotaxis operons encoding homologues of the *E. coli* chemotaxis proteins (Szurmant and Ordal, 2004). The chemotaxis operons in these organisms can regulate flagellar motility, or other cellular functions (Masduki *et al*., 1995; Kato *et al*., 1999; D'Argenio *et al*., 2002; Ferrandez *et al*., 2002; Kirby and Zusman, 2003; Berleman *et al*., 2004; Vlamakis *et al*., 2004; Berleman and Bauer, 2005a). In *Rhodobacter sphaeroides,* chemosensory components from two operons are required for chemotaxis (Porter *et al*., 2002; Porter and Armitage, 2004) and mutations in both operons leads to a discrete motility bias and impaired chemotaxis. In this species, it was proposed that chemosensory processing originating in two chemotaxis pathways may be required to reach a threshold level of phosphorylated CheY, sufficient to effectively modulate the rotational bias of the flagellar motor and mediate optimum chemotaxis (Porter and Armitage, 2002). In *Pseudomonas aeruginosa*, components from three clusters that comprise genes encoding two full sets of chemotaxis protein homologs contribute to optimum chemotaxis and mutants in only one gene cluster have a discernible motility bias (Kato *et al*., 1999; Ferrandez *et al*., 2002).

It is possible that Che2 directly modulates *R. leguminosarum* chemotaxis only under specific conditions that were not identified in this study. If Che2 functions to modulate chemotaxis under specific conditions, these conditions could be specified by the sensory abilities of MCPs. Interestingly, the sensory and signaling domains of the three MCP homologs encoded as part of the Che2 cluster do not have any homolog of

known or unknown function. Alternatively, the role of Che2 in chemotaxis may be secondary, and its primary function may be to control other cellular responses than motility.

It was previously shown in *E. coli* that cells with higher reversal frequencies tend to form swarms of larger diameters relative to cells with a random steady-state swimming bias in spatial gradient assays, whereas cells with smooth swimming biases tend to form swarms of smaller diameters (Wolfe and Berg, 1989). The steady-state smooth swimming bias of the Δ*che1* and the *Δche1,2* mutants is consistent with the formation of swarms of smaller diameter on all compounds tested in the spatial gradient assay for chemotaxis. Despite the observation that the Δ*che2* mutant constantly swam with a higher reorientation frequency relative to the parent strain, it did form smaller swarms, but only under certain conditions (pyruvate as the chemoeffector). These results indicate that under the conditions of the swarm plate assay, the Δ*che2* mutant relied solely on the components of *che1* for carrying out chemotaxis to some compounds (e.g. galactose) while components of the *che2* cluster may contribute to chemotaxis toward specific (but not all) chemoeffectors (e.g. pyruvate). MCP(s) that would preferentially and/or more efficiently interact with components of the *che2* cluster could account for such specificity (chemoeffector-dependent) in the contribution of *che2* to chemotaxis.

How then does $CheB_{II}$ contribute to chemotaxis in *R. leguminosarum* bv. *viciae* strain 3841 in the absence of other components of the *che2* cluster? Interestingly, the effect of deleting $cheB_{II}$ on the motility bias implies that the $cheB_{II}$ gene was expressed and its product functional in the Δ*che2* background. By analogy with other chemotaxis systems and because the presence of $CheB_{II}$ caused an increase in the probability of

changes in the swimming direction (Δ*che2* mutant), we hypothesize that $CheB_{II}$ may affect the methylation levels of MCPs that directly interact with the signaling activity of components of *che1*. Interestingly, CheB homologs from different chemotaxis pathways have been implicated in the ability of cells to modulate the signaling output from another major chemotaxis system in other bacterial species such as *Pseudomonas aeruginosa* (Ferrandez *et al*., 2002), *Rhodospirillum centenum* (Berleman and Bauer, 2005b) and *A. brasilense* (Stephens *et al*., 2006). Based on these data and the results obtained here for *R. leguminosarum*, we argue that CheB homologs from multiple chemotaxis-like clusters may function to modulate the signaling output from a major chemotaxis cluster. Such a role for multiple CheB homologs may allow cells to integrate sensory information about the environment from multiple pathways into a single output and coordinated response. Future experiments to elucidate the role of $CheB_{II}$ as well as other adaptation proteins in *R. leguminosarum* bv. *viciae* should be productive.

Our data reveal that *che1* from *R. leguminosarum* bv. *viciae* is homologous and closely related to the chemotaxis operon controlling flagellar motility in plant-associated alpha-proteobacteria *S. meliloti* and *A. tumefaciens.* The results of the nodulation competition experiments demonstrate that *che1* promotes competitive nodule formation and increases the fitness of *R. leguminosarum* bv. *viciae* strain 3841 in presence of the host plant, *Pisum sativum.* One interesting implication of this finding is that the environmental cue(s) triggering chemotaxis of motile soil *R. leguminosarum* bv. *viciae* cells toward the roots of pea and facilitating colonization are likely to be processed through the *che1* cluster. Based on these data and our phylogenetic analysis, we hypothesize that *che1* orthologs in the Rhizobiaceae may have evolved to promote

chemotaxis and plant association. This finding provides direct evidence for the role of a specific set of chemotaxis proteins in establishing a host-microbe association. Similarly, other studies have shown that specific chemotaxis genes promote host colonization and increase competitiveness. For example, several defined non-chemotactic mutants of *Helicobacter pylori* are less competitive than the wild type in infection of mice (Terry *et al*., 2005). Nodulation competition experiments in *R. leguminosarum* strain VF39SM have previously implicated two MCPs as being important for Pea nodulation (Yost *et al*., 1998). A mutant of *A. brasilense* lacking an energy-sensing MCP is severely affected in wheat root surface colonization (Greer-Phillips *et al*., 2004). Similarly, some chemotaxis genes of *Pseudomonas aeruginosa* were shown to be differentially regulated in response to root exudates of two varieties of sugar beets, further suggesting a role for chemotaxis in host specificity and selective plant root colonization (Mark *et al*., 2005). Finally, two MCP homologs are required for optimum colonization of the chick gastrointestintal tract by *Campylobacter jejuni* (Hendrixson and DiRita, 2004). Taken together, these data on different host-associated microorganisms clearly show that dedicated sets of chemotaxis genes promote competitive colonization of specific niches. Our comparison of the distribution of *R. leguminosarum* bv. *viciae che1* and *che2* orthologs in other alpha-proteobacteria and the comparison of their function also illustrate the fact that chemotaxis operons evolve rapidly, further strengthening the hypothesis for the role of this function in providing a competitive advantage and facilitating adaptation to specific environments.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids are listed in Table 2.4. *R. leguminosarum* strains were grown aerobically in TY medium (Beringer, 1974) or Vincent's minimal medium (VMM) (Vincent, 1970) at 28°C with shaking. *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C with shaking. Antibiotic concentrations used to culture *R. leguminosarum* were gentamicin 30 μg ml^{-1}, kanamycin 50 μg ml^{-1} and streptomycin 500 μg ml^{-1}. Antibiotic concentrations used to culture *E. coli* were gentamicin 15 μg ml^{-1} and kanamycin 50 μg ml^{-1}.

Mutant construction

Deletion of *che1* was performed as follows. A 1,093 bp region immediately upstream of *che1* and including 317 bp of the first gene in the operon was PCR amplified with the primer pair Op1upFwd (5'-GAGCTCGGCGGCTTGGAATAATAG) and Op1upRev Op1upRev (5'-TCTAGAATCTTGCTTTCGGTATCG) including a 5' *Sac*I site and a 3' *Xba*I site (engineered restriction sites are underlined). A 1,075 bp region beginning 513 bp from the end of the last gene in the operon, *cheD,* and extending downstream of the operon was PCR amplified with the primer pair Op1dwnFwd (5'-CTGCAGGGCGAGTACAAGGTTCTG) and Op1dwnRev (5'-GGGCCCCGCAAGAATAAGGAGAGA) including a 5' *Pst*I site and a 3' *Apa*I site (engineered restriction sites are underlined). These fragments were cloned into the vector pCR®2.1-TOPO® using the TOPO TA cloning® kit (Invitrogen) according to the

Table 2.4. Strains and plasmids used in this study[a].

Strain or plasmid	Genotype, phenotype or description	Reference or source
Strains		
R. leguminosarum:		
3841	biovar *viciae*, JB300 derivative Sm^r	Johnston and Beringer (1975); Glenn *et al*. (1980)
LM100	Δ*che1*, Km^r, Sm^r	this study
LM200	Δ*che2,* Sm^r	this study
LM300	Δ*che1,2*, Km^r, Sm^r	this study
LM400	Δ*che2cheB$_{II}$*, Gm^r, Sm^r	this study
LM500	3841 containing pFUS1, Sm^r, Tc^r	this study
LM600	3841 containing pFop1, Sm^r, Tc^r	this study
LM700	3841 containing pFop2, Sm^r, Tc^r	this study
LM800	3841 containing pML122, Gm^r, Km^r	this study
LM900	3841 containing pML*cheB$_{II}$*, Gm^r, Km^r	this study
LM1000	LM400 containing pML122, Gm^r, Km^r	this study
LM1100	LM400 containing pML*cheB$_{II}$*, Gm^r, Km^r	this study
E. coli:		
DH5α	General cloning strain	Gibco BRL
S17-1	Sm^r/Sp^r; RP4 *tra* region, mobilizer strain	Simon *et al*. (1983)

TOP10	General cloning strain	Invitrogen
Plasmids		
pCR®2.1-TOPO®	PCR cloning vector, Km^r	Invitrogen
pCM184	Km^r	Marx and Lidstrom, (2002)
pFUS1	pMP220 derivative containing a promoterless *gusA,* Tc^r	Reeve *et al*. (1999)
pFUS1op1up	pFUS1, op1up fragment from TopoOp1up inserted as an *Eco*RI fragment	this study
pFUS1op2up	pFUS1, op2up fragment from TopoOp2up inserted as an *Eco*RI fragment	this study
pIJ9861	pLAFR1 cosmid carrying 3841 genes RL0679-RL700	J.A. Downie
pIJ9776	pLAFR1 cosmids carrying 3841 genes RL4020-4044	J.A. Downie
pRK2013	helper plasmid with RK2 tra region	Figurski and Helinski, (1979)
pJQ200SK	Suicide vector for gene replacement, Gm^r	Quandt and Hynes,(1993)
pJQLM1	Deletion construct for *che1*, Gm^r	this study
pJQLM2	Deletion construct for *che2*, Gm^r	this study
pJQLM2b	Deletion construct for $cheB_{II,}$ Gm^r	this study
pML122	Expression vector, *mob/rep,* Gm^r, Km^r	Labes *et al.*, (1990)
pML$cheB_{II}$	pML122 containing $cheB_{II}$, Gm^r, Km^r	this study

TopoOp1up	pCR®2.1-TOPO® containing the PCR product from the primer pair Op1upFwd and Op1upRev, Km^r	this study
TopoOp1dwn	pCR®2.1-TOPO® containing the PCR product from the primer pair Op1dwnFwd and Op1dwnRev, Km^r	this study
TopoOp2up	pCR®2.1-TOPO® containing the PCR product from the primer pair Op2upFwd and Op2upRev, Km^r	this study
TopoOp2dwn	pCR®2.1-TOPO® containing the PCR product from the primer pair Op2dwnFwd and Op2dwnRev, Km^r	this study
Topo*cheB$_{II}$*	pCR®2.1-TOPO® containing the PCR product from the primer pair cheB$_{II}$Fwd and cheB$_{II}$Rev, Km^r	this study

[a] Gm^r gentamicin resistance, Km^r kanamycin resistance, Sm^r streptomycin resistance, Sp^r spectinomycin resistance, Tc^r tetracycline resistance.

manufacturer's instructions. The resulting plasmids were termed TopoOp1up and TopoOp1dwn. The fragments were then excised with the restriction enzymes *Sac*I and *Xba*I for the upstream fragment and *Pst*I and *Apa*I for the downstream fragment and inserted into the suicide vector pJQ200SK (Quandt and Hynes, 1993) at the appropriate restriction sites to generate the deletion construct. A cassette encoding resistance to kanamycin was isolated from pCM184 and cloned into the *Sma*I site of the deletion construct, pJQLM1. The deletion construct was inserted into strain 3841 by allelic exchange as described in Quandt and Hynes (1993). The resulting strain was termed LM100.

Deletion of *che2* was performed as follows. A 1,247 bp region immediately upstream of *che2* and including 24 bp of the first gene in the operon was PCR amplified with the primer pair Op2upFwd (5'-CGAGCTCCGAGGTGCGTGGCGAAAC)and Op2upRev (5'- GTCTAGAGGTGGTCAGACGAATGCTG) to include a 5' *Sst*I site and a 3' *Xba*I site (engineered restriction sites are underlined). A 1,037 bp region beginning within the second to last gene in the operon, *cheR,* and extending downstream to include a portion of the last gene in the operon, *cheB,* was PCR amplified with the primer pair Op2dwnFwd (5'-GTCGACTGGCGATGGTGCTGGCGG) and Op2dwnRev (5'-GGGCCCAGCATCACATCCGCCGTC)including a 5' *Sal*I site and a 3' *Apa*I site (engineered restriction sites are underlined). These fragments were cloned into the vector pCR®2.1-TOPO® using the TOPO TA cloning® kit and the resulting plasmids were termed TopoOp2up and TopoOp2dwn. These fragments were then excised with the restriction enzymes *Sst*I and *Xba*I for the upstream fragment and *Sal*I and *Apa*I for the downstream fragment and cloned into the suicide vector pJQ200SK at the appropriate

restriction sites to generate the deletion construct, pJQLM2. The deletion construct was inserted into strain 3841 by allelic exchange as described in Quandt and Hynes (1993). The resulting strain was termed LM200. A mutant lacking both *che1* and *che2*, LM300, was generated by recombining the mutated *che1* carried on pJQLM1 into strain LM200. Deletions were verified by both PCR and Southern blot analysis.

To delete $cheB_{II}$ in the Δ*che2* background, the suicide vector pJQ200SK containing the Op2down fragment was modified by digestion with *Bam*HI and re-ligated in order to delete the sequence with homology to *cheR* from the Op2down fragment leaving only an internal 368 bp fragment of $cheB_{II}$. This construct, pJQLM2b, was inserted into Δ*che*2 via bi-parental mating using *E. coli* S17.1 as the donor strain as described in Quandt and Hynes (1993). Complementation of the mutants was carried out by introducing the cosmids pIJ9861 and pIJ9776 by conjugation using pRK2013 as a helper plasmid. These two cosmids and information as to their content, were generously supplied by Dr. Allan Downie, John Innes Institute, Norwich, UK. pIJ9861 carries all the genes of the *che1* operon and pIJ9776 contains the entire *che2* cluster (J.A. Downie, personal communication).

β-glucuronidase assays

Sequences upstream of the first gene in the *che1* and *che2* cluster were cloned into the pFUS1 promoter probe vector, which contains a promoterless *gusA* gene (Reeve *et al.,* 1999) in order to assess the activity of corresponding promoter. The TopoOp1up and TopoOp2up vectors that each carry a DNA sequence spanning a region upstream of and including the 5' end of the first gene of each *che* cluster were digested by *Eco*RI to

release the insert. Gel-purified fragments were then cloned into the *EcoRI*–linearized pFUS1 vector. The proper orientation of the insert relative to the promoterless gusA gene was verified by restriction analysis. The resulting plasmids, pF*che1* and pF*che2*, along with pFUS1 were inserted individually into *R. leguminosarum* strain 3841, via conjugation using the donor strain *E. coli* S.17.1 as previously described (Quandt and Hynes, 1993), yielding strains LM500, LM600, and LM700. The β-glucuronidase activity was measured from cultures grown aerobically at 28°C with shaking in either TY medium or VMM with different carbon sources added from 200 mM stocks in chemotaxis buffer (K_2HPO_4, 10 mM; KH_2PO_4, 10 mM; EDTA, 0.1 mM [pH 7.0]) and containing tetracycline 20 μg ml^{-1} for plasmid maintenance. Cultures were grown to mid to late exponential phase and motility was verified by inspecting cultures visually with a dark field microscope before harvesting 3-4 ml by centrifugation (3,500 rpm, 3 min; eppendorf centrifuge 5417R). The supernatant was removed and cell pellets were stored at -80°C until *β*-Glucuronidase activity was measured using the method described by Reeve *et al.* (2002).

Electron microscopy

R. leguminosarum bv. *viciae* was grown overnight with shaking at 28 °C in TY to mid-logarithmic phase. Cultures were inspected to ensure motility, and cells were taken directly from the culture and adsorbed to Formvar-coated nickel grids (EM sciences) for 1 min. Excess culture was blotted with Whatman filter paper and the grid was placed face down on a drop of 1% uranyl acetate (EM Sciences) for 1 min. Excess stain was

blotted with Whatman filter paper, and the grid was air-dried and viewed with an LEO 906e transmission electron microscope operating at an accelerating voltage of 80 kV.

Behavioral assays

Swarm plates were prepared using VMM without carbon source amended with agar (FisherBiotech) at 0.2% (wt/v). Carbon sources were added from 200 mM stocks in chemotaxis buffer (K_2HPO_4, 10 mM; KH_2PO_4, 10 mM; EDTA, 0.1 mM [pH 7.0]) to a final concentration of 1 mM. The number of cells in the inoculum was standardized by using a 5 µl aliquot of cells in exponential phase adjusted to the same OD_{600} value. Plates were incubated at 28°C for 5 days after which the diameter of the swarm rings was measured. The mutant swarm sizes were expressed as a percent relative to the parent. Each carbon source was tested four times from two independent cultures.

To determine the reorientation frequency of each strain, cultures were grown to mid-exponential phase in VMM and their swimming behavior was recorded using a video camera attached to a dark field microscope. Video recordings were manually analyzed in blind experiments by counting the number of changes in swimming direction undergone by individual cells per second and averaging the response of at least 30 cells each from three independent cultures.

Swimming paths were obtained with the Hobson BacTracker (Hobson Tracking Systems, Sheffield, UK) by analyzing video recordings of motile cultures of *R. leguminosarum* strains grown in VMM pyruvate 10 mM to mid-exponential growth phase.

To determine the rotational bias of *R. leguminosarum* flagella, motile cultures were grown to mid-logarithmic phase in VMM mannitol 10 mM. Suspensions were observed

through a Nikon Eclipse E200 phase contrast microscope with a 100X oil immersion objective lens. Video recordings were made of cells found to be tethered by their flagella to the microscope slide. Videos of tethered cells were analyzed with CellTrak motion analysis software from Motion Analysis Corp. to determine the change in direction of orientation over time. A total of 10 cells from 3 independent cultures was analyzed for about 10 seconds each. Graphs shown are from representative cells.

Nodulation assays

Nodulation assays and competition assays of pea (*Pisum sativum* variety Trapper) were carried out essentially as described in Yost et al. (1998). Briefly, surface-sterilized seedlings were co-inoculated with parental strain 3841 and either the Δ*che1* or Δ*che2* or Δ*che1,2* or Δ*che2cheB*$_{II}$ mutants in approximate 1:1 and 1:10 ratios. Complemented Δ*che1* was treated similarly. The exact proportion of parental to mutant strains was confirmed by viable plate counts on the inoculant suspensions. Bacteria were re-isolated from surface sterilized nodules after 3-4 weeks of plant growth and identified based on antibiotic resistance markers. Controls included uninoculated plants, and pea plants inoculated by each individual strain. Controls were examined for plant appearance, nodule number and appearance, and plant aerial matter was dried and weighed to compare total dry matter production, which is a measure of nitrogen fixation in plants grown in N-free medium.

Comparative genomic analysis

The complete genome sequence of *R. leguminosarum* bv. *viciae* strain 3841 was determined by the Wellcome Trust Sanger Institute (Young *et al*., 2006). We searched

the genome using tblastn with the amino acid sequences of CheA from the close relatives *Agrobacterium tumefaciens* and *Sinorhizobium meliloti.* Nucleotide sequences exhibiting homology were further analyzed using FramePlot, an open reading frame prediction tool developed specifically for bacteria with high G+C content and available at http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl. (Ishikawa and Hotta, 1999). Predicted protein sequences were analyzed using SMART, a protein domain architecture predicting tool available at http://smart.embl-heidelberg.de/ (Letunic *et al.* 2006) and PSI-BLAST (Altschul *et al*., 1997) searches against the non-redundant database at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nih.gov).

Phylogenetic analysis of α-proteobacterial chemotaxis operons

DNA sequences of the 16S rRNA gene were retrieved from the non-redundant database at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nih.gov). Sequences were aligned using CLUSTAL X (Thompson *et al*. 1997). A phylogenetic tree was built from the CLUSTAL alignment using the neighbor joining method. Orthology relationships between homologs of chemotaxis operons were determined by comparing sequence similarity and gene order, as previously described (Hauwaerts *et al.*, 2002). Gene order was determined using the Microbial Signal Transduction database (MiST; http://genomics.ornl.gov/mist) (Ulrich *et al.,* 2005).

Statistical analysis

Statistical analyses for behavioral and expression assays was performed in Microsoft Excel. A Student's *t*-test assuming equal variances ($P < 0.05$) was used to determine

significant differences between strains. Statistical analyses for nodulation competition experiments were performed in Microsoft Excel. A chi-square test was used to determine if there was a significant difference between inoculation and recovery ratios ($P < 0.001$ and $P < 0.05$ were tested).

ACKNOWLEDGMENTS

The authors want to thank the Motion Analysis Corporation for providing access to the beta version of the CellTrak software, Sandy Parkinson and Allan Downie for strains and plasmids, and Bonnie Stephens for assistance with TEM. This work was supported in part by a NSF CAREER award to G. A. (MCB-0622277) and NSERC discovery grants to C. K. Y. and M. F. H.

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CHAPTER 3

INTRODUCTION

Motile bacteria sense their environment and navigate chemical gradients to position themselves in optimum niches for growth and metabolism via a process termed chemotaxis (Armitage, 1999; Wadhams and Armitage, 2004). Environmental stimuli are sensed by chemoreceptors, so-called methyl-accepting chemotaxis proteins or MCPs, and this signal is transduced to a two-component system the output of which interacts with the flagellum thereby regulating motility (Armitage, 1999; and Wadhams and Armitage, 2004).

Typical MCPs are situated in the cytoplasmic membrane. They possess N-terminal sensing domains that protrude into the periplasm and cytoplasmic C-terminal signaling domains that transduce signals to the two-component system controlling chemotaxis (Falke and Hazelbauer, 2001; Zhulin, 2001). Some MCPs, such as the aerotaxis transducer Aer of *Escherichia coli*, are membrane bound but have a cytoplasmic sensing domain (Bibikov *et al*., 1997, Rebbapragada *et al*., 1997). Still others such as TlpC from *Rhodobacter sphaeroides* localize to the cytoplasm and are thought to process signals related to the cell's metabolic state (Wadhams *et al*., 2002).

Bacteria have evolved an extraordinarily diverse array of MCP sensory modules allowing them to sense and respond to such diverse physico-chemical parameters as chemical ligands, light, temperature, redox value, pH, and oxygen (Zhulin, 2001). Aerotaxis is the process by which bacteria navigate gradients of oxygen. Bacteria have

evolved several different ways to perform this advantageous behavior including binding oxygen directly and sensing other physiological parameters that are affected by oxygen such as changes in either the electron transport system, ETS, or the proton motive force (Taylor *et al*., 1999).

Aerotaxis via energy taxis

Aerotaxis has been the subject of much investigation in *E. coli.* In *E. coli*, two separate MCPs mediate the aerotaxis response albeit by different mechanisms: Aer and Tsr (Bibikov *et al*., 1997, Rebbapragada *et al*., 1997, Greer-Philips *et al*., 2003). As mentioned, Aer is membrane bound, but both its N-terminal sensory domain and C-terminal signaling domain are cytoplasmic (Bibikov *et al*., 1997). Its sensory domain bears an FAD binding PAS domain allowing it to monitor changes in the electron transport system, ETS, by undergoing changes in redox potential (Bibikov *et al*., 1997, Rebbapragada *et al*., 1997, Taylor and Zhulin, 1999; Bibikov *et al*., 2000; Repik *et al*., 2000; Greer-Philips *et al*., 2003). Alternatively, Tsr is a typical ligand-binding MCP that senses serine but also has the ability to sense intracellular energy levels and therefore transduces oxygen, redox, and energy signals (Rebbapragada *et al*., 1997; Greer-Philips *et al*., 2003).

Aerotaxis by direct oxygen sensing

Chemoreceptors able to directly bind and therefore sense diatomic oxygen were first described in *Bacillus subtilis* and *Halobacterium salinarum* (Hou *et al*., 2000). The

receptors were dubbed HemATs (HemAT-*Bs* and HemAT-*Hs*) for heme-containing aerotaxis transducer (Hou *et al*., 2000).

Several lines of evidence demonstrate that these receptors are oxygen binding heme-containing MCPs. First, the sequences of the N-terminal sensory domains of these receptors display homology to sperm whale myoglobin and possess the absolutely conserved heme-binding proximal histidine residue in the F helix (F8) and phenylalanine in the CD region (Bashford *et al*., 1987; Hou *et al*., 2000). Alanine replacement mutagenesis of each histidine in the heme-binding region of HemAT for both species confirmed that the proximal histidine in the F helix binds heme (Hou *et al*., 2001). Second, the proteins exhibit spectral properties characteristic of heme proteins (Hou *et al.*, 2000). Third, a pyridine haemochrome assay showed that the heme groups are of the b-type (Hou *et al*., 2000). Finally, mutant strains lacking the HemAT receptor were affected in their behavioral response to oxygen in a capillary assay (Hou *et al*., 2000). Interestingly, HemAT apparently mediates an aerophobic response in *H. salinarum* and an aerophilic response in *B. subtilis*.

The heme-binding sensory domain comprises a functional domain of prokaryotic signal transducers that bind heme in a globin fold and is part of globin-coupled sensor (GCS) motif (Hou *et al*., 2001). Freitas *et al.* (2003) describe a large GCS superfamily whose members can be divided into aerotactic and gene regulating subfamilies depending on the C-terminal transmitter domain.

Freitas *et al*., (2003) describe the criteria for identifying a putative GCS: a primary match with the globin domain, the length of the globin domain, and the presence of a proximal histidine. Predicted secondary structure was also used to support inclusion

of a protein and almost all of the GCS proteins identified using these criteria also had a hydrophobic aromatic residue pair (usually Phe-Tyr) at the end of the predicted B helix (Freitas *et al*., 2003). The conserved domain database at NCBI recognizes the heme-binding globin domain present in GCSs as sensor_globin.

Variation of the sensor_globin domain

Rhizobium leguminosarum is an α-proteobacterium that lives planktonically in the soil but also has the ability to form an endosymbiotic relationship with its host plant *Pisum sativa*, the pea plant. A recent study found that of its two che clusters *che1* is the major che cluster responsible for chemotaxis and is required for competitive nodulation of *P. sativa* (Miller *et al*., 2007). In addition to the chemotaxis proteins cheABDRWY$_I$Y$_{II}$, *che1* encodes a putative chemoreceptor (Miller *et al*., 2007). Because *che1* is required for both chemotaxis and competitive nodulation we initiated this study to characterize the chemoreceptor it encodes.

A CD-search (Marchler-Bauer and Bryant, 2004) revealed the presence of a sensor_globin domain at the N-terminus of this putative chemoreceptor. However, further analysis including a multiple sequence alignment of the sensory domain of MCPs with N-terminal sensor_globin domains revealed that the sensor_globin domain lacked the proximal histidine residue required for heme-binding. This analysis revealed three other putative chemosensory proteins that possess a putative sensor_globin domain but lack a proximal histidine residue. The closely related organisms *Agrobacterium tumefaciens, Rhizobium etli,* and *Sinorhizobium meliloti* each encode a single protein that meets these criteria. In *S. meliloti*, this protein has been termed IcpA for internal

chemotaxis protein. For this reason we have designated its homolog in *R. leguminosarum* IcpA-*Rl*.

In order to determine the function of IcpA-*Rl* we generated a mutant strain of *R. leguminosarum* deleted for *icpA*-Rl and analyzed its behavior. In addition, we cloned and expressed IcpA-*Rl* under native conditions and analyzed the absorption spectra to determine if the protein binds heme. We found that Δ*icpA*-Rl was unaffected in its aerotaxis response but was affected in its chemotactic ability. Purified IcpA-*Rl* did not exhibit absorption spectra characteristic of oxygen-bound heme proteins. We therefore hypothesize that IcpA-*Rl* is an energy sensing chemotaxis protein and represents a novel function of the sensor_globin domain.

RESULTS AND DISCUSSION

Identification of a variation of the sensor_globin domain

We have identified four proteins in the closely related species *A. tumefaciens*, *R. etli*, *R. leguminosarum*, and *S. meliloti* that are predicted to encode sensor_globin domains coupled to an MCP signaling domain. Proteins with a sensor_globin domain at the N-terminus coupled to a methyl-accepting, or MA, domain at their C-terminus are members of a superfamily of proteins called globin coupled sensors, GCSs (Freitas et al., 2003). These proteins are referred to as HemAts for heme-binding aerotaxis transducers. Despite the presence of a sensor_globin domain at their N-terminus, a multiple sequence alignment revealed that these four proteins do not contain either the proximal histidine residue or the hydrophobic pair at the end of the B helix (Figure 3.1). Therefore, these

A

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Rleg_YP_766295     37 RNRPALEAHLKAGLRDLFHRFQTFPDASRN  66
Retl_YP_468179.1   33 RNRPALKAHLKAGLRDLFHRFQSYPDAARN  62
Atum_NP_353543.1   85 NYRGMLEPYVKAGLRDVMTRFQSMPDCSPS 114
Smel_NP_384742.1   36 DHRQALSPRIELALRALSHRLQASPDAARH  65
Ssp_YP_614641.1    36 GMAPLVERHIDAVLDDFYDLCLSRPETKGY  65
Ssp_YP_613971.1    24 KRAKMILGLLPEVLDHFYDRVGREPEMAAF  53
Goxy_YP_191196.1   28 RIRPQALGVLGPALDRFYGRIHKTPALNRF  57
Rsph_YP_353306.1   25 ATRALLNEAVGRALDRFHERMRQT-SAAGF  53
Atum_NP_354049.1   30 DMKGVITGSLDASLDRFYTKVRAVPETAKF  59
Ccre_NP_421120     67 AIKPVIDAEIGAALGQFYSQVRLFPDTRVK  96
Ccre_NP_419247     30 DLRPVIRAEIGKALDNFYGKVRATPETRKF  59
Rleg_YP_766036     29 DLRPVISELIGGALDKFYAKIAKTPAVAGF  58
Retl_YP_467960.1   54 DLSPVISELIGAALDKFYAKVARTPAVSGF  83
Zmob_YP_162617.1   24 ALKKIVERHGQKTLDYLYTHLTRTS-AARF  52
Bsub_NP_388919.1   53 QLQPLIQENIVNIVDAFYKNLDHESSLMDI  82
Hsp_NP_280321.1    52 AEQPLFEATADALVTDFYDHLESYERTQDL  81
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B

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Rleg_YP_766295     96 YAERVKVLSDTESKMGLDPRWHVAGHGVML 125
Retl_YP_468179.1   92 YAERVKVLSDTESKMGLDPRWHVAGHGVML 121
Atum_NP_353543.1  144 YAERVKVLSDNAGRMGLDPRWQIASHAVVL 173
Smel_NP_384742.1   95 YAERVKVLADTESRMGLDPRWQIASHAIVL 124
Ssp_YP_614641.1    95 YMSSADKVGRVHFEVDLPFHLFLGGYATVG 124
Ssp_YP_613971.1    83 YIQSARNIGRVHTRIGLPFSFFNAGYAHAN 112
Goxy_YP_191196.1   87 YVESTRAIGNTHARIGLKPQWYVGGYALLI 116
Rsph_YP_353306.1   83 YVEEAVRVGRTHARIGLEPRWYLGGYALIL 112
Atum_NP_354049.1   89 YTNAVTAIGRTHARLGLEPRWYIGGYALML 118
Ccre_NP_421120    126 YVRDVERIGRSHADADIAPQWYIGGYAVVV 155
Ccre_NP_419247     89 YVQAVRAIGQTHARIGLEPRWYIGGYAVVG 118
Rleg_YP_766036     88 YVNGVTAVGRTHARIGLEPRWYIGGYAIVM 117
Retl_YP_467960.1  113 YVDGVTAVGRTHARIGLEPRWYIGGYALVM 142
Zmob_YP_162617.1   82 SEETSKKIGAIHAQIGVTPTYYISAYAVNL 111
Bsub_NP_388919.1  112 FIEKRNRIASIHLRIGLLPKWYMGAFQELL 141
Hsp_NP_280321.1   112 YAAQRARIGKIHDVLGLGPDVYLGAYTRYY 141
```

Figure 3.1 An alignment of the sensory domains of MCPs from α-proteobacteria containing sensor_globin domains at their N-terminal sensory domain focusing on the region surrounding A) the conserved pair of hydrophobic residues at end of the B helix and B) the proximal histidine residue required for heme-binding. Amino acid sequences of the well characterized HemATs from *Bacillus subtilis* and *Halobacterium salinarum* were included as controls. NCBI accession numbers for each sequence are listed beside the species abbreviations. Species abbreviations: Atum, *Agrobacterium tumefaciens*; Bsub, *Bacillus subtilis*; Ccre, *Caulobacter crescentus*; Goxy, *Gluconobacter oxydans*; Hsp, *Halobacterium sp*. NRC-1, Retl, *Rhizobium etli*; Rleg, *Rhizobium leguminosarum*; Rsph, *Rhodobacter sphaeroides*; Smel, *Sinorhizobium meliloti*; Ssp, *Silicibacter* TM1040; Zmob, *Zymomonas mobilis*.

proteins must either bind heme in a way different from the previously characterized GCSs or not at all.

As with the HemATs, none of these proteins contain detectable transmembrane regions and are thus predicted to be cytoplasmic. For this reason, in *S. meliloti*, this protein was termed, IcpA, for internal chemotaxis protein A, (Meier *et al*., 2007). In *S. meliloti,* IcpA is the only MCP of its nine to possess a sensor_globin domain. In contrast, *A. tumefaciens*, *R. etli*, and *R. leguminosarum* encode both a HemAT homolog and an IcpA homolog.

A phylogenetic tree based on the multiple-sequence alignment of the sensor_globin domains of MCPs from α-proteobacteria reveals a distinct branch bearing the sequences from *A. tumefaciens*, *R. etli*, *R. leguminosarum*, and *S. meliloti* that lack both the proximal histidine and the hydrophobic pair at the end of the B helix suggesting that these closely related proteins are homologs (Figure 3.2).

Of the four putative MCPs we identified bearing sensor_globin domains that lack a proximal histidine, *S. meliloti* IcpA is the best studied. The authors of a recent study by Meier *et al*. (2007), generated an *S. meliloti* mutant strain deleted for the gene encoding IcpA, *icpA,* and analyzed its behavior. Of the nine chemoreceptor-deleted strains analyzed Δ*icpA* exhibited the greatest impairment in chemotaxis in a spatial gradient assay (Meier *et al*., 2007). Δ*icpA* also exhibited a significant reduction in chemokinesis and was strongly impaired in chemotaxis to proline in a capillary assay (Meier *et al*., 2007). Based on their results, the authors hypothesize that IcpA is a sensor for the metabolic state of the cell (Meier *et al*., 2007).

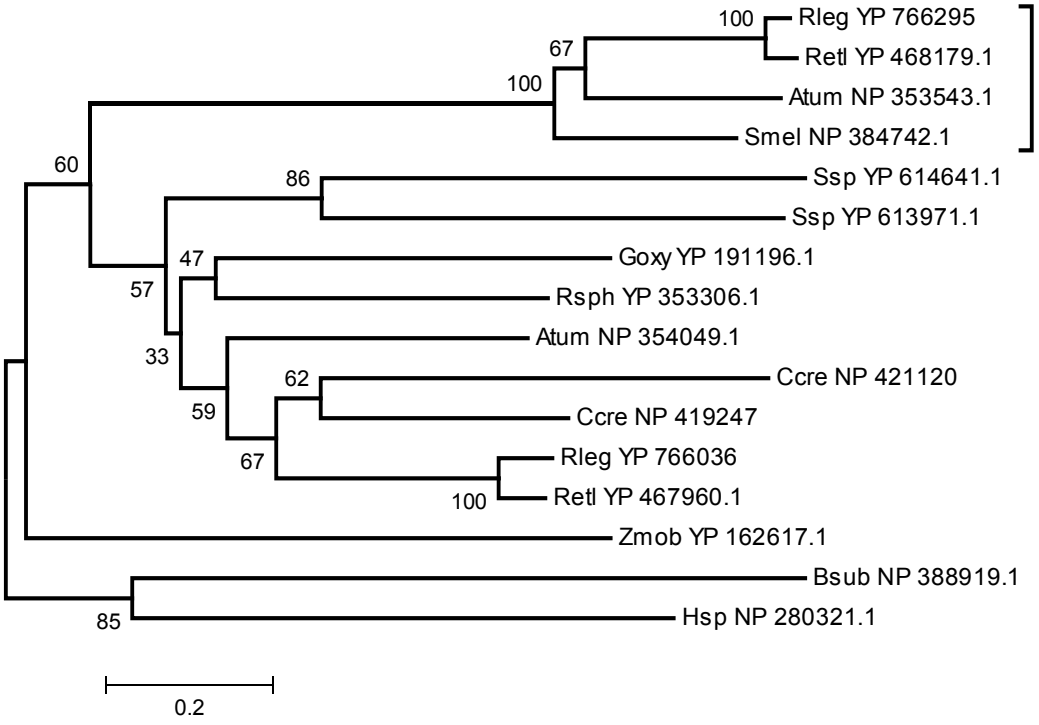

Figure 3.2 A phylogenetic tree of the sensor_globin domain from MCPs of α-proteobacteria. The structural alignment was built using T-Coffee and the tree was built using the neighbor joining method. The bracket indicates a group of homologs that lack both the proximal histidine residue and the hydrophobic pair at the end of the B helix. NCBI accession numbers for each sequence are listed beside the species abbreviations. Species abbreviations: Atum, *Agrobacterium tumefaciens*; Bsub, *Bacillus subtilis*; Ccre, *Caulobacter crescentus*; Goxy, *Gluconobacter oxydans*; Hsp, *Halobacterium sp*. NRC-1, Retl, *Rhizobium etli*; Rleg, *Rhizobium leguminosarum*; Rsph, *Rhodobacter sphaeroides*; Smel, *Sinorhizobium meliloti*; Ssp, *Silicibacter* TM1040; Zmob, *Zymomonas mobilis.*

Given the absolute requirement of a proximal histidine for covalent binding of the heme cofactor (Bashford *et al*., 1987) and the evidence suggesting that IcpA is an energy sensor we hypothesize that these receptors are not heme containing aerotaxis transducers but instead represent a novel function of the sensor_globin domain in sensing energy related parameters in chemotaxis.

IcpA homologs do not bind heme

In order to determine if *S. meliloti* IcpA and IcpA-*Rl* bind heme we cloned and expressed these proteins along with HemAT-*Rl* under native conditions. The purified recombinant HemAT-*Rl* displayed absorption spectra that are typical of oxygen-bound heme proteins whereas IcpA and IcpA-Rl did not (Figure 3.3). HemAT-Rl exhibited an absorption maxima of 415 nm (Soret), 575 nm (α-band), and 540 nm (β-band) compared to corresponding maxima of 406, 578, and 538 nm for HemAT-*Bs* and HemAT-*Hs* (Hou *et al*., 2000). In contrast, IcpA and IcpA-*Rl* did not exhibit absorption spectra characteristic of oxygen-bound heme proteins (Figure 3.3).

Upon deoxygenation with sodium dithionite, the Soret bands of HemAT-Rl shift to 430 nm, and the α- and β-bands converge to a single peak at 560 nm (Figure 3.4). This is consistent with the shift seen in the absorption bands for HemAT-*Bs* and HemAT-*Hs*: 425 nm and 555 nm (Hou et al., 2000). Deoxygenation had no effect on the absorption spectra of IcpA and IcpA-*Rl* (Figure 3.4).

Given that recombinant HemAT-*Rl* was able to bind heme, this observation suggests that IcpA and IcpA-*Rl* do not covalently bind a heme cofactor. We therefore

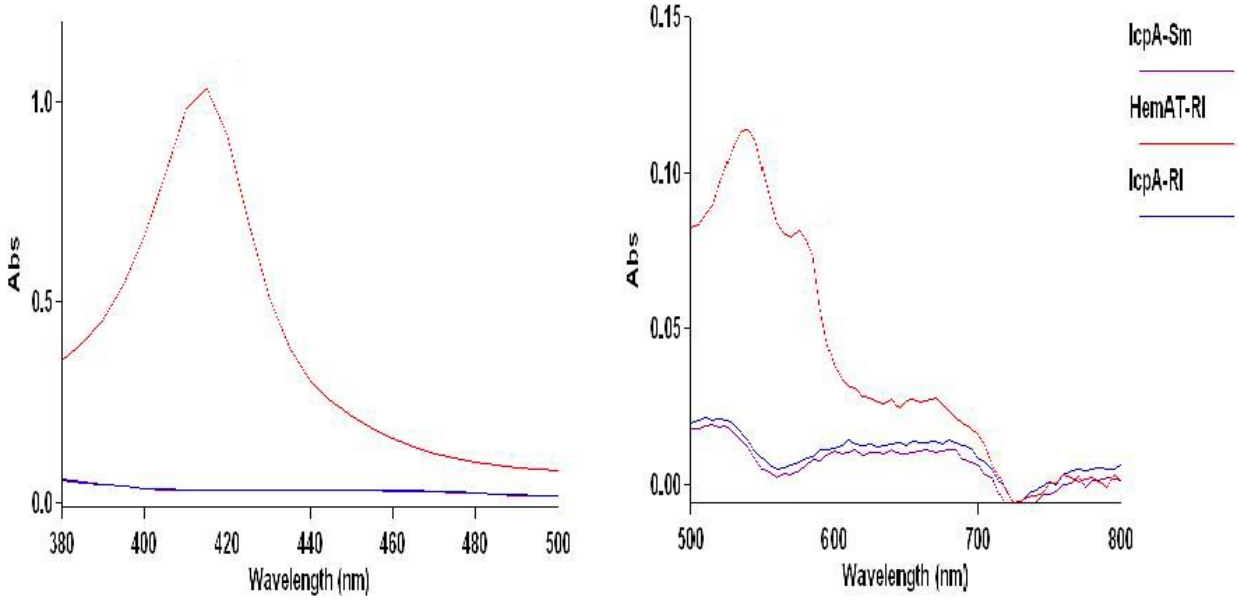

Figure 3.3 Absorption spectra of purified oxygenated IcpA-*Sm*, HemAT-*Rl*, and IcpA-*Rl*. HemAT-Rl exhibits absorption spectra characteristic of oxygen-bound heme proteins whereas IcpA-*Sm* and IcpA-*Rl* do not.

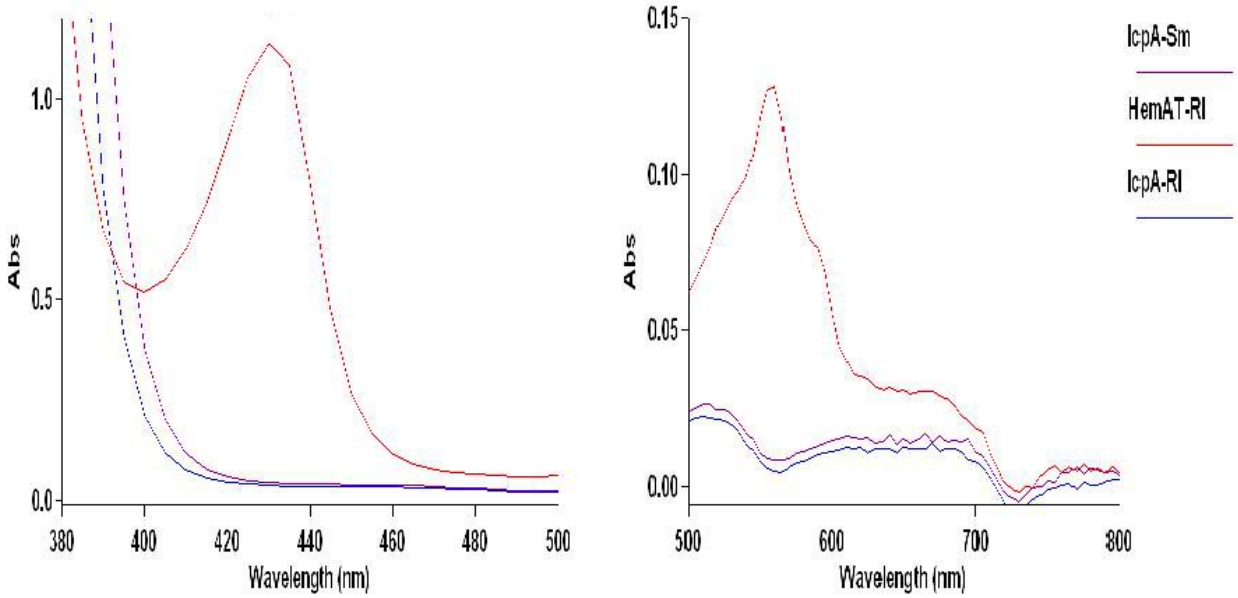

Figure 3.4 Absorption spectra of purified IcpA-*Sm*, HemAT-*Rl*, and IcpA-*Rl* deoxygenated with sodium dithionite. HemAT-*Rl* exhibits a shift in absorption maxima consistent with the formation of a deoxy form. In contrast deoxygenation has no effect on either IcpA-*Sm* or IcpA-*Rl*.

hypothesize that the sensor_globin domain of these proteins utilizes a yet to be determined cofactor either covalently as with the HemATs or non-covalently as with the FAD binding PAS domain of *E. coli* Aer (Bibikov *et al*., 1997; Bibikov et al., 2000; Freitas et al., 2003).

IcpA-Rl is deficient in chemotaxis but not aerotaxis

We generated a mutant deleted for the gene encoding IcpA-Rl, Δ*icpA*-Rl, and analyzed its swimming behavior and chemotactic ability. We measured the reorientation frequency of Δ*icpA*-Rl as previously described (Miller *et al.*, 2007) and found it to be statistically the same as the parent strain (Figure 3.5). We analyzed the chemotactic ability of Δ*icpA*-Rl using the spatial gradient assay, or swarm plate assay, and found it to be impaired under all conditions tested (Figure 3.6). The behavior of Δ*icpA*-Rl is similar to that of *S. meliloti* Δ*icpA* reported by Meier *et al*., (2007) and supports the hypothesis that IcpA-*Rl* is an energy sensor.

In order to determine the contribution of IcpA-*Rl* to the *R. leguminosarum* aerotaxis response, we first characterized the response of the parent strain to temporal changes in oxygen concentration using the micro-slide chamber assay previously described by Laszlo and Taylor, 1981. When exposed to a decrease in oxygen concentration *R. leguminosarum* responds by increasing its reorientation frequency (Figure 3.7). This increased reorientation frequency continues without adaptation for several minutes. Upon return of oxygen cells resume the pre-stimulus reorientation frequency. Because cells respond to the removal of oxygen and not the addition, we have termed this response "anaerophobic." A Δ*che1,2* mutant lacking both che clusters (Miller

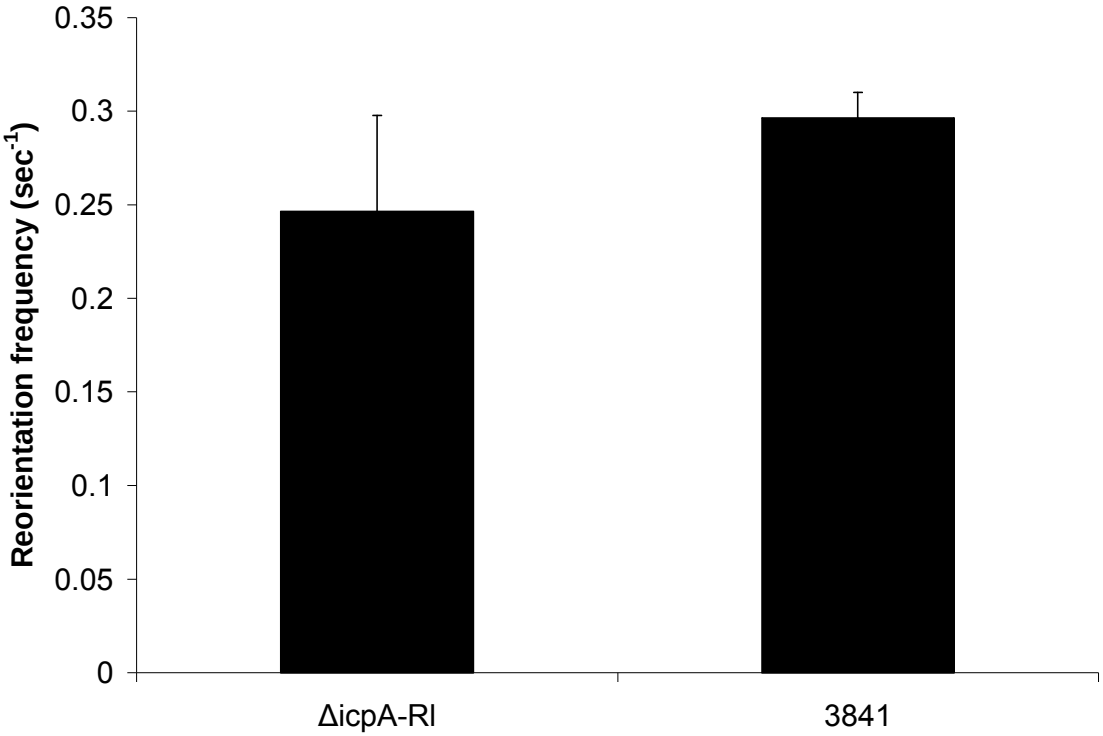

Figure 3.5 The motility bias of Δ*icp*-Rl is the same as the parent strain 3841. Cells were grown in minimal media with mannitol 10 mM and the reorientation frequency was measured the as described in Experimental procedures. There was no statistical difference between the reorientation frequencies based on a Student's *t*-test ($P < 0.05$).

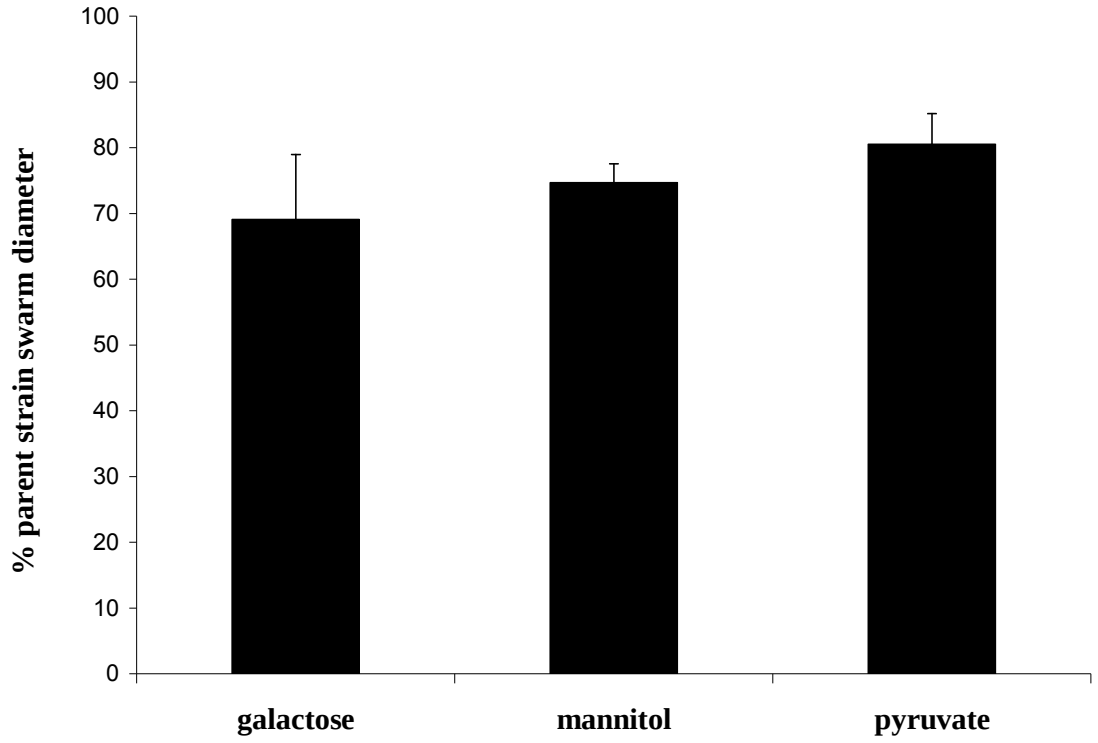

Figure 3.6 Δ*icpA*-Rl is impaired in chemotaxis. A spatial gradient assay was used to test chemotaxis to various carbon compounds at 1 mM. Error bars represent the standard deviations from at least three replicates. The Δ*icpA*-Rl swarm diameters were statistically different from the parent strain as determined by a Student's *t*-test ($P < 0.05$).

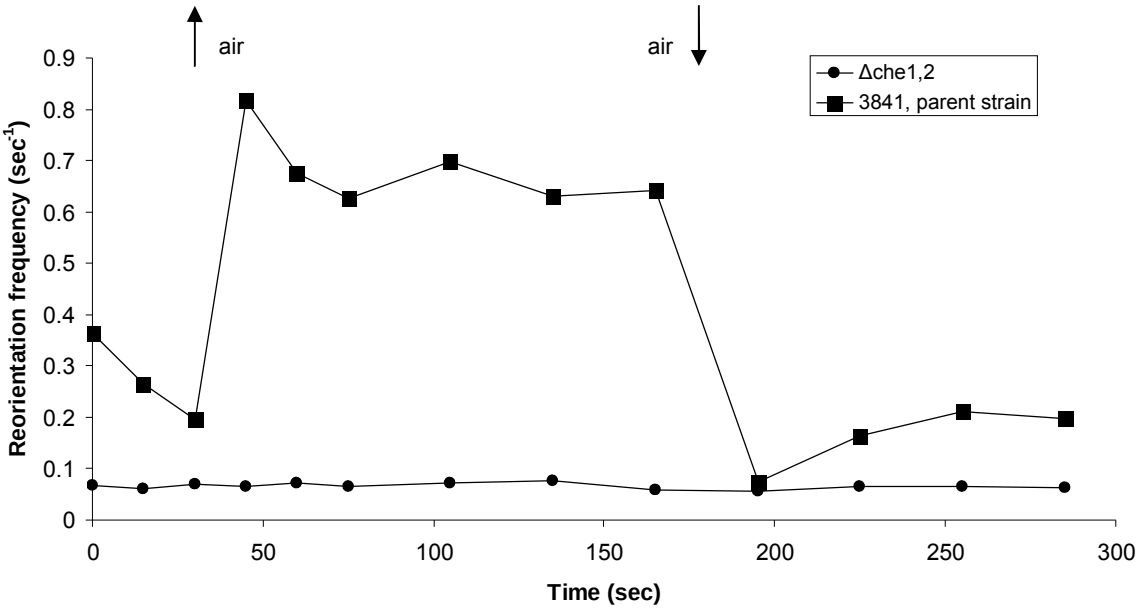

Figure 3.7 The aerotaxis response of *R. leguminosarum* is dependent on an intact chemotaxis system. Upon removal of oxygen, *R. leguminosarum* increases its reorientation frequency. This increased reorientation frequency continues without adaptation for several minutes. Upon return of oxygen, *R. leguminosarum* resumes its pre-stimulus motility bias. A Δ*che1,2* mutant lacking both che clusters did not respond to either removal or addition of air. Each point represents the average reorientation frequency of ten cells. Similar results were obtained in at least two additional replicates.

et al., 2007) showed no response to either removal or addition of oxygen indicating that an intact chemotaxis system is required for the response (Figure 3.7).

Δ*icpA*-Rl responds to a decrease in oxygen concentration by increasing its reorientation frequency similar to the parent strain (Figure 3.8). Upon return of oxygen Δ*icpA*-Rl returns to its pre-stimulus reorientation frequency similar to the parent strain. These results indicate that IcpA-*Rl* is not involved in the *R. leguminosarum* anaerophobic response. However, since *R. leguminosarum* also encodes a HemAT it is possible that HemAT can compensate for the loss of IcpA-*Rl*. Efforts are currently underway in order to determine the contribution of HemAT to the *R. leguminosarum* anaerophobic response and whether the presence of HemAT masks the effects of deleting IcpA-*Rl*.

Based on our results and data from the literature, we propose that IcpA-*Rl* is an energy taxis transducer and represents a novel function of the sensor_globin domain. The molecular mechanism of energy sensing for IcpA-*Rl* and whether any cofactors are required has yet to be determined. Efforts are currently underway to further characterize the function of IcpA and IcpA-*Rl* by testing the taxis responses to respiratory inhibitors.

Energy taxis in micro-organisms is likely to be widespread and the ecological significance of this type of behavior is only beginning to be understood (Alexandre *et al*., 2003). Greer-Philips *et al.* (2004) described a novel energy taxis transducer, Tlp1, in *Azospirillum brasilense* that is important for root colonization. Miller *et al.* (2007) demonstrated the requirement of *R. leguminosarum che1,* the major chemotaxis gene cluster*,* for competitive nodulation of its host plant. Given that IcpA-*Rl* is encoded by che1 it is possible that IcpA-*Rl* is important for efficient nodulation of the host plant. Efforts are currently underway to test this possibility. Further research on IcpA-Rl and

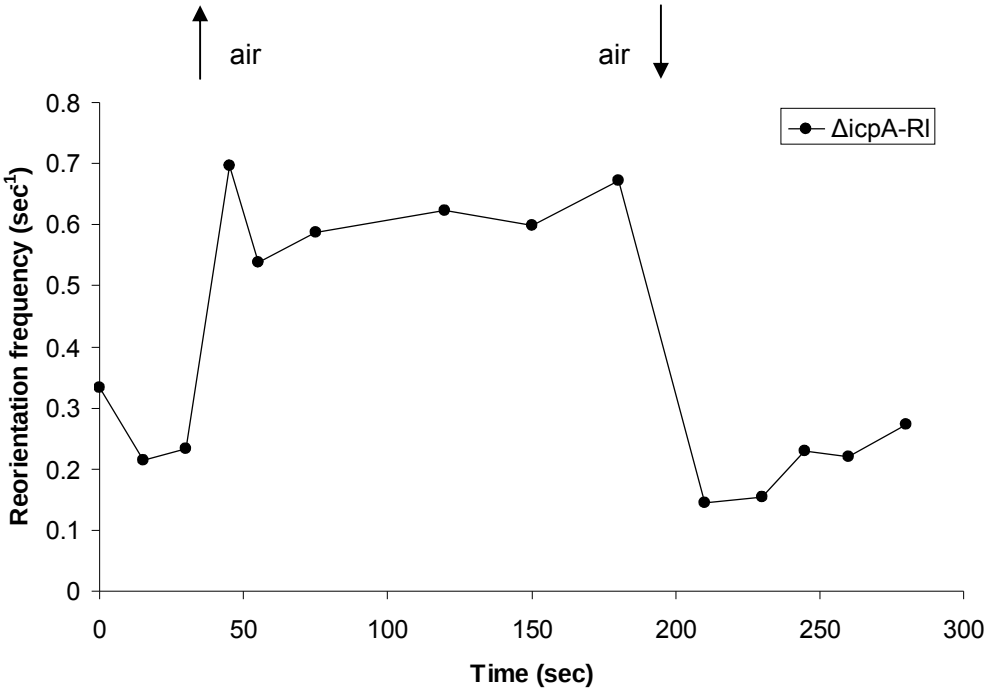

Figure 3.8 The aerotaxis response of Δ*icpA*-Rl is similar to the parent strain. Upon removal of oxygen, Δ*icpA*-Rl increases its reorientation frequency. This increased reorientation frequency continues without adaptation for several minutes. Upon return of oxygen, Δ*icpA*-Rl resumes its pre-stimulus motility bias. Similar results were obtained in at least two additional replicates.

IcpA will contribute to our knowledge of energy taxis transducers and the ecological role of this process.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids are listed in Table 3.1. *E. coli* strains were grown in either Luria-Bertani (LB) medium or Terrific broth (TB) at 37°C with shaking. *R. leguminosarum* strains were grown aerobically in TY medium (Beringer, 1974) or Vincent's minimal medium (VMM) (Vincent, 1970) at 28°C with shaking. *S. meliloti* strains were grown in TYC (Platzer *et al.*, 1997). Antibiotic concentrations used to culture *R. leguminosarum* were gentamicin 30 μg ml^{-1}, kanamycin 50 μg ml^{-1} and streptomycin 500 μg ml^{-1}. Antibiotic concentrations used to culture *E. coli* were gentamicin 15 μg ml^{-1} and kanamycin 50 μg ml^{-1}.

Multiple alignment and phylogenetic analysis

Protein sequences were procured from the Microbial Signal Transduction Database (Ulrich and Zhulin, 2007) and were analyzed for the presence of sensor_globin domains by performing BLAST searches against the non-redundant database and the microbial database at the National Center for Biotechnology Information (http://www.ncbi.nig.gov/BLAST/).

Table 3.1 Strains and plasmids used in this study[a].

Strain or plasmid	Genotype, phenotype or description	Reference or source
Strains		
Escherichia coli:		
BL21(DE3)	F$^-$ *ompT hsdS$_B$* (r_B^- m_B^-) *gal dcm* (DE3)	Invitrogen
Mach1^{TM}-T1^R	F$^-$ Φ*80lac*ZΔM15 *Δlac*X74 *hsd*R(r_K^- m_K^-) *Δrec*A1398 *end*A1 *ton*A	Invitrogen
DH5α	General cloning strain	Gibco BRL
S17-1	Sm^r/Sp^r; RP4 *tra* region, mobilizer strain	Simon *et al*. (1983)
TOP10	General cloning strain	Invitrogen
Rhizobium leguminosarum:		
3841	biovar *viciae*, JB300 derivative Sm^r	Johnston and Beringer (1975); Glenn *et al*. (1980)
LM300	Δ*che1,2*, Km^r, Sm^r	Miller *et al*. (2007)
LH100	Sm^r; Δ*icpA-Rl*	this study
Sinorhizobium meliloti:		
RU11/001	Sm^r; spontaneous streptomycin-resistant wild-type strain	Pleier and Schmitt (1991)

Table 3.1 (continued)

Plasmids:		
pCR®2.1-TOPO®	PCR cloning vector, Km^r	Invitrogen
pET SUMO	Km^r; protein expression vector	Invitrogen
pET-*hemAT*	1497 bp PCR fragment containing *hemAT* cloned into pET SUMO	this study
pET-*icpA-Rl*	1635 bp PCR fragment containing *icpA-Rl* cloned into pET SUMO	this study
pET-*icpA-Sm*	1602 bp PCR fragment containing *icpA-Sm* cloned into pET SUMO	this study
pJQ200SK	Suicide vector for gene replacement, Gm^r	Quandt and Hynes, (1993)
pJQ-icpA-Rl	deletion construct for *icpA-Rl*, Gm^r	
pRK2013	helper plasmid with RK2 *tra* region	Figurski and Helinski, (1979)
TopoOp1up	pCR®2.1-TOPO® containing the PCR product from the primer pair Op1upFwd and Op1upRev, Km^r	this study
TopoIcpAdwn	pCR®2.1-TOPO® containing the PCR product from the primer pair IcpAdwnFwd and IcpAdwnRev, Km^r	this study

[a] Gm^r gentamicin resistance, Km^r kanamycin resistance, Sm^r streptomycin resistance, Sp^r spectinomycin resistance, Tc^r tetracycline resistance.

Sequences were aligned using T-Coffee (Notredame *et al*., 2000), and a phylogenetic tree was built from the alignment using the neighbor joining method in MegAlign.

DNA methods

R. leguminosarum and *S. meliloti* DNA was isolated and purified with a Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA). Plasmid DNA was purified with a FastPlasmid™ Mini Kit (Eppendorf, USA) and DNA fragments or PCR products were purified from agarose gels using a QIAquick Gel Extraction Kit (Qiagen, Maryland, USA).

Mutant construction

Deletion of *icpA*-Rl was performed as follows. A 1,093 bp region immediately upstream of *che1* and including 317 bp of the *icpA*-Rl gene in the operon was PCR amplified with the primer pair Op1upFwd (5'-GAGCTCGGCGGCTTGGAATAATAG) and Op1upRev (5'-TCTAGAATCTTGCTTTCGGTATCG) including a 5' *Sac*I site and a 3' *Xba*I site (engineered restriction sites are underlined). An 835 bp region beginning 45 bp before the end of the *icpA* gene was PCR amplified with the primer pair IcpA-RlDwnC (5'-CTGCAGCCGCAAGCGTTCGCAAGC) and IcpA-RlDwnD (5'-GGGCCCCTGTTCCTCGCACTCCTG) including a 5' *Pst*I site and a 3' *Apa*I site (engineered restriction sites are underlined). These fragments were cloned into the vector pCR®2.1-TOPO® using the TOPO TA cloning® kit (Invitrogen) according to the manufacturer's instructions. The resulting plasmids were termed TopoOp1up and IcpA-Rldwn. The fragments were then excised with the restriction enzymes *Sac*I and *Xba*I for

the upstream fragment and *Pst*I and *Apa*I for the downstream fragment and inserted into the suicide vector pJQ200SK (Quandt and Hynes, 1993) at the appropriate restriction sites to generate the deletion construct. The deletion construct was inserted into strain 3841 by allelic exchange as described in Quandt and Hynes (1993). The resulting strain was termed LH100.

Expression and purification of HemAT-Rl, IcpA-Rl and IcpA-Sm

Recombinant HemAT-*Rl* was overproduced from plasmid pET-*hemAT*, IcpA-*Rl* from pET-*icpA Rl,* and IcpA from pET-*icpA Sm* in *E. coli* BL21(DE3) (Table 3.1). Cells were grown at 37°C in TB containing 50 µg ml^{-1} kanamycin to an OD_{600} of 0.7 and gene expression was induced by 0.5 mM IPTG. Cultivation was continued for 16 h at room temperature until harvest. Cells from a 2 L culture were resuspended in 20 ml lysis buffer [50 mM NaH_2PO_4, 300 mM NaCl, 1 mM PMSF, 10 mM Imidazol; pH 8.0] containing benzonase and lysed by sonication. Cell membranes and debris were removed by centrifugation at 15 000 rpm and 4°C for 30 min. The supernatant was loaded on a Ni-NTA (Qiagen, Maryland , USA) column equilibrated with lysis buffer and washed thoroughly with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 1 mM PMSF, 20 mM Imidazol; pH 8.0) at 4°C.

Spectrophotometric assays

Spectrophotometric assays were conducted in a Cary 50 UV-visible Spectrophotometer (Varian) at room temperature. Absorbance spectra between 380 nm and 800 nm were recorded by scanning 100 µg of the purified proteins HemAT, IcpA-*Rl* and IcpA-*Sm*

solved in 1 ml cleavage reaction buffer [25 mM Tris-HCl (pH 8.0), 150 mM $NaCl_2$, 1 mM DTT, 5% Glycerol] against the appropriate buffer. Deoxygenation was achieved by the addition of a few grains of sodium dithionite ($Na_2S_2O_4$) to 1 ml of each protein solution.

Behavioral assays

Swarm plates were prepared using VMM without carbon source amended with agar (FisherBiotech) at 0.2% (wt/v). Carbon sources were added from 200 mM stocks in chemotaxis buffer (K_2HPO_4, 10 mM; KH_2PO_4, 10 mM; EDTA, 0.1 mM [pH 7.0]) to a final concentration of 1 mM. The number of cells in the inoculum was standardized by using a 5 µl aliquot of cells in exponential phase adjusted to the same OD_{600} value. Plates were incubated at 28°C for 5 days after which the diameter of the swarm rings was measured. The mutant swarm sizes were expressed as a percent relative to the parent. Each carbon source was tested four times from two independent cultures.

To determine the reorientation frequency of each strain, cultures were grown to mid-exponential phase in VMM and their swimming behavior was recorded using a video camera attached to a bright field microscope. Video recordings were manually analyzed in blind experiments by counting the number of changes in swimming direction undergone by individual cells per second and averaging the response of at least 30 cells each from three independent cultures.

Aerotaxis experiments were performed using the microslide chamber assay described by Laszlo and Taylor, (1981). Briefly, a 30 µl aliquot of a bacterial culture grown in TY liquid broth to OD_{600} 0.6 was placed on a microscope slide and placed into the microslide chamber. The microslide chamber was ventilated with either atmospheric air or nitrogen gas from compressed gas cylinders.

Statistical analysis

Statistical analyses for behavioral assays were performed in Microsoft Excel. A Student's *t*-test assuming equal variances ($P < 0.05$) was used to determine significant differences between the means.

ACKNOWLEDGEMENTS

We thank Frauke Purschke for her work cloning and expressing *S. meliloti* IcpA and HemAT-*Rl* and for performing spectroscopy analysis of purified proteins. We thank Latifa Hamzaoui for her work generating the IcpA-*Rl* mutant. We thank Birgit Scharf for the generous gift of *S. meliloti* strains.

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CHAPTER 4

CONCLUDING REMARKS

Rhizobium leguminosarum bv. *viciae* is an endosymbiotic bacterium that is a major contributor to the globally important process of nitrogen fixation. While chemotaxis was previously implicated in establishing the relationship between *R. leguminosarum* and its host *Pisum sativa* the exact role of chemotaxis in establishing the *R. leguminosarum*-legume symbiosis was unclear. When this study to characterize the chemotaxis system of *R. leguminosarum* was initiated, little was known about motility in *R. leguminosarum*, and the chemotaxis system of *R. leguminosarum* had not been described.

The availability of the *R. leguminosarum* genome enabled us to identify and describe the chemotaxis gene clusters of *R. leguminosarum* and to design constructs for the creation of genetically defined chemotaxis mutants. We analyzed the behavior of these chemotaxis mutants in order to determine the contribution of each *che* cluster to the behavior of *R. leguminosarum* and to determine the role of chemotaxis in competitive nodulation of the host plant. The major findings of this study are:

I. Motility— *R. leguminosarum* was found to possess one to two subpolar flagella. Motion analysis of tethered cells suggests that *R. leguminosarum* regulates its motility by either varying the speed of flagellar rotation or briefly pausing rotation.

II. Two *che* clusters—*R. leguminosarum* was found to encode two clusters of chemotaxis genes, *che1* and *che2*, that have the characteristics of operons. Expression studies using promoter fusions to the β-glucuronidase gene found that both *che* clusters are expressed under all conditions tested.

III. *che1* and *che2* contribute to chemotaxis—*che1* was found to be the major che cluster that regulates motility. *che2* was found to play a minor role in chemotaxis possibly by contributing the output of *che1* via $CheB_{II}$.

IV. *che1* is required for competitive nodulation (but not nodulation per se)—It was clearly demonstrated using genetically defined mutants and *in vivo* nodulation competition experiments that *che1* is required for competitive nodulation of *Pisum sativa* by *R. leguminosarum*.

V. Aerotaxis—*R. leguminosarum* exhibits an aerotactic response to temporal changes in oxygen concentration. This response was characterized by an increase in reorientation frequency in response to decreases in oxygen concentration in a temporal assay and is therefore termed an "anaerophobic" response. The absence of this anaerophobic response in a Δ*che1,2* mutant that lacks both *che* clusters indicates that this response requires an intact chemotaxis system.

VI. Characterization of IcpA-*Rl*—An MCP encoding a variation of the globin coupled sensor domain was identified and characterized. A mutant lacking IcpA-*Rl*, Δ*icpA*-Rl, was generated and its behavior was analyzed. Δ*icpA*-Rl was impaired in chemotaxis, but motility bias and aerotaxis response were not affected. The absence of the conserved histidine residue in the globin coupled

sensor domain of IcpA-*Rl* and the defect in chemotaxis of the Δ*icpA*-Rl mutant suggests a novel function of the globin coupled sensor domain.

The availability of the *R. leguminosarum* bv. *viciae* strain 3841 genome (Young *et al*., 2006) allowed for the identification of components of the chemotaxis system of *R. leguminosarum*. The two *che* clusters are homologous to chemotaxis operons that control flagellar motility and chemotaxis in other organisms (Greck *et al*., 1995; Sourjik and Schmitt, 1996; Hamblin *et al.*, 1997). Therefore, the role of each *che* cluster in the chemotaxis pathway of *R. leguminosarum* cannot be predicted from sequence information alone.

Interestingly, this study demonstrates a role for both *che* clusters in the chemotactic response of *R. leguminosarum*. While multiple chemotaxis operons have been shown to regulate chemotaxis in other organisms this is the first demonstration of two *che* clusters contributing to chemotaxis in a rhizobial species (Kato *et al*., 1999; Ferrandez *et al*., 2002; Porter and Armitage 2002, 2004).

However, as is the case with much of science, each answer raises still more questions. Are the components of each *che* cluster separated spatially within the cell? Are each of the 27 MCPs dedicated to a particular pathway or are they able to signal through both *che* clusters? This study demonstrates that $CheB_{II}$ from *che2* contributes to the signaling output of *che1*. Does this interaction takes place at the level of the receptors? Are the CheA proteins from each pathway able to phosphorylate response regulators encoded by the other pathway?

Studies using protein fusions to fluorescent proteins and *in vitro* biochemical studies on purified proteins would begin to address questions of localization and the

degree to which cross-talk occurs. However, a larger question is perhaps more difficult to address: Do the two *che* clusters regulate other cellular processes besides chemotaxis? The adaptation process that enables the temporal sensing mechanism of chemotaxis by allowing the sensory status of chemoreceptors to be reset and therefore sensitive to small changes in ligand concentration over a wide range of concentrations also makes this system an ideal candidate for processing signals for other cellular processes. Examples of cellular processes that are regulated by chemotaxis pathways in other organisms include developmental programs and expression of flagella (Kirby and Zusman, 2003; Berleman *et al*., 2004; Vlamakis *et al*., 2004; Berleman and Bauer, 2005a, 2005b). More work is required to determine if either of the *che* clusters is involved in the regulation of cellular processes besides chemotaxis in *R. leguminosarum*.

This study demonstrates that *che1* is required for competitive nodulation of the host plant *Pisum sativa* suggesting an important role for chemotaxis and the *che1* cluster in *R. leguminosarum*'s lifestyle. However, it is unclear whether chemotaxis to a specific chemical signal is involved in chemotaxis to the roots or if the bacteria are simply attracted to the rhizosphere because of the many chemoattractants such as amino acids, organic acids, and sugars that support the growth of rhizobia and are present in plant root exudates (Brencic and Winans, 2005).

Establishment of the *R. leguminosarum*-legume symbiosis requires the exchange of numerous chemical signals between the host plant and the bacterium (Oke and Long, 1999). Previous studies demonstrated chemotaxis by *R. leguminosarum* to the nod factors apigenin and naringenin (Armitage *et al*., 1988). However, the concentrations of these compounds that were found to elicit chemotactic responses were quite low and

could be overwhelmed by other components of root exudates. Yost *et al*. (1998) demonstrated that mutants deleted for individual chemoreceptors were impaired in competitive nodulation suggesting that these MCPs sense chemoattractants that promote chemotaxis towards the plant roots. However, specific chemical ligands have not been identified for these MCPs.

A search of the *R. leguminosarum* genome revealed 27 genes encoding putative MCPs. Identification of ligands for these MCPs will be a major area for future research on chemotaxis in *R. leguminosarum.* This study has contributed to this pursuit by characterizing the MCP IcpA-*Rl.* The authors of a study of MCPs in *S. meliloti* hypothesized that a homolog of IcpA-*Rl* senses the metabolic state of cell (Meier *et al*., 2007). The evidence presented here supports this hypothesis and demonstrates that receptor represents a novel function of the globin coupled sensor domain and likely evolved from a gene duplication event of the gene encoding the heme-binding aerotaxis protein HemAT. However, the exact function of IcpA-*Rl* remains to be determined. Efforts are currently underway to characterize the function of IcpA-*Rl* using metabolic inhibitors and determine whether it contributes to the ability of *R. leguminosarum* to compete for nodulation opportunities.

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